DEC 14 1938
MEDICAL LIBRARY

VOL. 24 DECEMBER, 1938 No. 3

The Journal of Laboratory and Clinical Medicine

WARREN T. VAUGHAN, M.D., EDITOR
201 W. Franklin St.
Richmond, Va.

ASSOCIATE EDITORS

Pharmacology
DENNIS E. JACKSON, M.D.
University of Cincinnati, Cincinnati

Tuberculosis
GERALD B. WEBB, M.D.
Cragmor Sanatorium, Colorado Springs

Clinical Pathology
W. C. MacCARTY, M.D.
Mayo Clinic, Rochester, Minn.

T. B. MAGATH, M.D.
Mayo Clinic, Rochester, Minn.

Biochemistry
VICTOR C. MYERS, Ph.D.
Western Reserve University, Cleveland

Experimental Medicine
RUSSELL L. HADEN, M.D.
Cleveland Clinic, Cleveland

Immunology
JOHN A. KOLMER, M.D.
Temple University, Philadelphia

ROBERT A. KILDUFFE, M.D.
Atlantic City, N. J.

Internal Medicine
GEORGE HERRMANN, M.D.
University of Texas, Galveston

Bacteriology
M. H. SOULE, Sc.D.
University of Michigan, Ann Arbor

Metabolism
CLIFFORD J. BARBORKA, M.D.
Northwestern University, Chicago

Surgery
DEAN LEWIS, M.D.
Johns Hopkins University, Baltimore

PUBLISHED BY THE C. V. MOSBY COMPANY, 3523-25 PINE BLVD., ST. LOUIS

Copyright 1938 by The C. V. Mosby Company

TABLE OF CONTENTS ON INSIDE COVER

The Journal of Laboratory and Clinical Medicine

CONTENTS FOR DECEMBER, 1938

Clinical and Experimental

Indications for Coramin in Cardiovascular Disease. Joseph Henry Cowan, M.D., Jersey City, N. J. ---- 225

Determination and Quantitative Estimation of the Decomposition of Chlorophyll in the Human Body. Joachim Theodor Brugsch, M.D., and Charles Sheard, Ph.D., Rochester, Minn. ---- 230

Prontosil and the Treatment of Spreading Peritonitis in Dogs. John O. Bower, M.D., and John C. Burns, M.D., Philadelphia, Pa., and Harold A. Mengle, M.D., Franklin, N. C. ---- 240

The Action of Chemical and Physical Agents on Clostridium Welchii and Its Toxin. Francis E. Colien, Ph.D., Omaha, Neb. ---- 245

The Role of the Upper Gastrointestinal Tract in the Etiology of Pernicious Anemia. W. H. Bachrach, B.S., and S. J. Fogelson, M.D., Chicago, Ill. 249

Studies in Convulsant Therapy. II. The Role of Alkalinization. Stanley Rochelle Dean, M.D., Newtown, Conn. ---- 256

Abnormal Uterine Bleeding as a Symptom in Typhoid Fever. Charles P. Wofford, M.D., Cleveland, Ohio, and Duncan G. Calder, M.D., and Ferdinand Fetter, M.D., Philadelphia, Pa. ---- 260

Dissecting Aneurysms of Aorta. Herbert J. Schattenberg, M.D., and Joseph Ziskind, M.D., New Orleans, La. ---- 264

A Standardized Procedure for the Study of Coagulation Reactions (In Vitro). John H. Ferguson, M.D., Ann Arbor, Mich. ---- 273

Multiple Primary Malignant Tumors. J. D. Kirshbaum, M.D., M.S., and F. L. Shively, Jr., B.S., B.M., Chicago, Ill. ---- 283

Laboratory Methods

The Quantitative Unreliability of the Nitroprusside Test for -SH and -S.S-. Frederick S. Hammett, Ph.D., and Sidney S. Chapman, B.S., North Truro, Mass. ---- 293

Reliability of the Agglutination Test for Undulant Fever. Howard J. Shaughnessy, Ph.D., Chicago, Ill., and Thomas C. Grubb, Ph.D., Baltimore, Md. 298

The Iron of Human Blood Serum. Burnham S. Walker, M.D., Boston, Mass. 308

Modified Apparatus for Obtaining Gastric Contents. Jules L. Posner, B.S., John T. Myers, Ph.D., M.D., and Andrew R. Fodor, B.A., New York, N. Y. ---- 315

The Determination of Morphine in the Urine of Morphine Addicts. Fred W. Oberst, M.S., Ph.D., Lexington, Ky. ---- 318

Department of Reviews and Abstracts

Selected Abstracts ---- 330

Book Reviews ---- 334

Vol. 24, No. 3, December, 1938 Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3523 Pine Blvd., St. Louis, Mo. Subscription Price: United States, its Possessions, Pan-American Countries, Canada, $8.50 a year; Foreign, $9.50. Entered as Second-Class Matter at Post Office at St. Louis, Mo., under Act of March 3, 1879.

The Journal of Laboratory and Clinical Medicine

VOL. 24 DECEMBER, 1938 NO. 3

CLINICAL AND EXPERIMENTAL

INDICATIONS FOR CORAMIN IN CARDIOVASCULAR DISEASE

JOSEPH HENRY COWAN, M.D., JERSEY CITY, N. J.

IN AN earlier investigation,[1] several observations were made in the treatment of a varied group of cardiac conditions. When this study was conducted, particular attention was paid to the use of the same drug, coramin, in the treatment of a group of patients in the so-called middle age period and decades subsequent, who showed varying degrees of impaired myocardial efficiency explainable on an arteriosclerotic and atherosclerotic basis.

There remains to be written a new chapter in medicine, dealing with this clinical entity. This type of degenerative myocardial disease has been described as (1) the failing heart of middle age; (2) chronic coronary artery disease, and (3) under a syndrome as multiple myocardial infarction. Correlated clinical and autopsy findings on these cases of hypertensive degenerative heart disease, giving histories of long periods of progressively impaired myocardial efficiency, bear out the pathologic patterns of numerous small myocardial infarctions resulting from multiple small closures with resulting diffuse replacement fibrosis.

Clinically, the patient referred to, is a sthenic individual, more frequently a male, in the beginning of the sixth decade, with an essentially negative past history, or a variable history of earlier hypertension, who has begun to notice breathlessness on exertion, discomfort over the sternum, varying from a feeling of uneasiness and pressure, to a stenocardia, occasional attacks of so-called indigestion or acute indigestion, fullness and belching after meals or on exertion, and lacking the potentialities and dynamics of a year or six months earlier. This individual may continue along under a reduced program of activity, which he

Received for publication, March 16, 1938.

may occasionally formulate and adjust himself to, and maintain his social and economic status reasonably well. Aside from dyspnea, many of these patients remain singularly free from symptoms.

Physical examination may reveal very few findings, or varying degrees of cardiac enlargement, a murmur of atherosclerotic aortic insufficiency, tortuous carotid vessels, a diastolic pressure over 90 mm. with a variable pulse pressure, hepatic enlargement, and diminished vital capacity. Radiologic changes may bear out these findings, with elongation and tortuosity of the arch and thoracic aorta, calcific deposits, and esophageal displacement. The electrocardiogram may be normal or show reduced voltage, T-wave changes, left ventricular preponderance, and ST or RT fusion.

In the beginning of these disturbances, there is a great discrepancy between the patients' subjective feelings and the objective findings of the physician, and there are probably more functional and physiologic changes than can be described by our clinical methods.

The structural change which has been described, resulting anatomically in a replacement fibrosis of the heart muscle, produces physiologically, states of myocardial asphyxia due to ischemia. Herein is traceable the succession of events, dependent on the alteration of coronary flow with altered cardiac muscle metabolism due to anoxic states. As organic changes advance and become permanent, the clinical picture advances with increasing grades of decompensation.

In the earlier investigations on coramin, electrocardiographically, one of the first changes produced was an increase in voltage, which sustained itself and which disappeared upon the withdrawal of the drug.

Coramin appears, therefore, to have a distinct influence on the heart in those patients who do not have any manifest central depression of respiration and circulation. It has been shown that it effects material differences in the electrical potentials of the heart's musculature. The question of how this effect is reached has been left open because there are many possibilities which cannot be exactly defined. It is definite that coramin improves the coronary flow by a vasodilatory effect or by some action on the extrinsic nervous mechanism of the heart as shown by some investigators, and by recent experiments of Stoland and Ginsberg[2] who demonstrated in the denervated heart-lung preparation that increase in the coronary flow takes place after a coramin injection. It is also quite possible that through the effect of coramin upon the respiratory and circulatory centers, there results a benefit to the circulation in general, improving the conditions for the efficiency of the actions of the heart. Either by a direct

Fig. 1.—MIA, MIIA, MIIIA: Syphilitic aortitis and aortic regurgitation. EKG taken May 13, 1936. MIB, MIIB, MIIIB: Patient B, EKG taken May 19, 1936, after treatment with coramin. KIIA: Patient K, EKG taken March 3, 1936. Neurocirculatory asthenia. KIIB: Patient K, EKG taken April 25, 1936. Continuously on coramin. PIA: Patient P, Normal heart, EKG taken March 24, 1936. PIB: Patient P, EKG taken March 28, 1936, showing voltage increase after administration of coramin. CIIA: Patient C, EKG taken April 22, 1936, patient receiving coramin and showing sinus rhythm. CIIB: Patient C, EKG taken May 11, 1936, showing arrhythmia possibly toxic in origin. FIIA: Patient F, EKG taken Feb. 26, 1936. Neurocirculatory asthenia. FIIB: Patient F, EKG taken March 30, 1936, after coramin administration. SIA, SIIA, SIIIA: Patient S, auricular fibrillation, EKG taken May 6, 1936. SIB, SIIB, SIIIB: Patient S, EKG taken May 18, 1936, continuously on coramin.

NOTE: In order to save space not all the leads were given in every case. The last letter, *A* and *B* respectively, indicates the electrocardiogram before and after treatment.

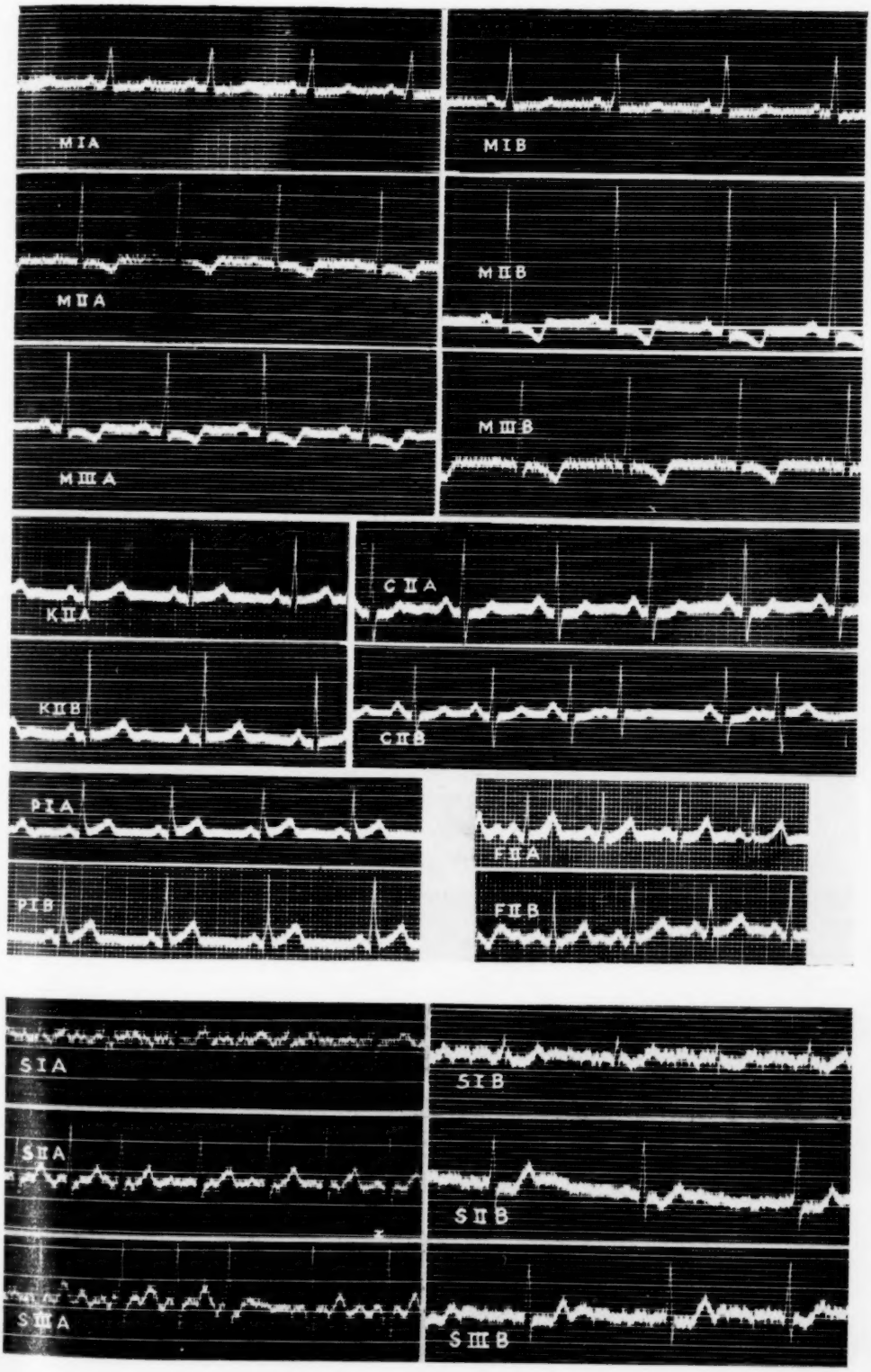

Fig. 1.—See bottom of opposite page for legend.

action or by the ways discussed, we have to imagine how coramin is effective in conditions which are characterized clinically by the syndrome described earlier. Improvements of venous return flow, vital capacity, and ventilation are probably all factors in the improvement of coronary flow and myocardial efficiency.

The conclusions of the earlier observations are briefly:

1. The margin of safety for coramin is wide, as regards the single dose or use over prolonged periods.

2. Electrocardiographically, the drug produces an increase in voltage for all the main ventricular complexes, an increase that is generally well sustained. There occurs at times, lesser and more variable changes in other components, such as the T-wave and the P-R interval. A few observations suggest that the A-V conductivity may be slightly depressed in patients with badly damaged myocardia.

3. Subjective and objective changes which pointed to increased myocardial efficiency after sufficient dosage with coramin. This improvement may be due to (a) an action on the extrinsic cardiac nerves, or (b) a direct action on the heart muscle, or (c) an increase in coronary flow.

Some graphs illustrating the electrocardiographic changes described are given in Fig. 1. They are selected from the material in my first investigation and from this paper. In every case all three leads were taken, although in order to save space only the one that shows the most characteristic but common change in these cases after the use of coramin has been reproduced in some instances.

Seventeen cases of chronic coronary artery disease were studied in this later observation. This number comprised a group of 13 white males and 4 white females. The ages ranged from 47 years to 79 years. The entire group was urban dwelling in character. Three of the women were married housewives; one was an unmarried successful business woman. Of the males, two were physicians, eight were business men, two were newspapermen, and one was a laborer. All of these cases showed evidence, clinically, functionally, and physiologically, of impaired myocardial efficiency. Dyspnea was a cardinal symptom, varying from breathlessness on considerable exertion to breathlessness and cyanosis at rest.

The following case histories are characteristic of those studied.

CASE 1.—E. V. L., male, aged 63 years, first noted breathlessness in December, 1936, on climbing steps. His attention was particularly attracted to this symptom because of the relatively long recovery period which brought him to his physician for examination. His past history was essentially negative, except that he was a voracious eater and inclined to worry. He had high blood pressure and was overweight. He was placed on a low calorie diet, given some digitalis, and told to ease up his activities. In January, 1937, he had several attacks of "asthma" during his sleep which necessitated his having an injection for relief. His examination on February 15, 1937, revealed: blood pressure 168/100, heart rate 96, regular and rhythmical, heart enlarged to the left and downward, sounds of distant character and embryocardic in nature, liver enlarged several centimeters below the costal margin, neck vessels distended and pulsating, pitting edema of the feet and ankles extending up to the middle of the tibiae.

Fluoroscopy revealed moderate enlargement of the inflow and outflow tracts of the left ventricle, elongation and tortuosity of the aortic arch, with displacement of the barium-filled esophagus to the right. EKG showed left axis deviation and high voltage. Bed rest for three weeks, restriction of fluids and diuresis established water balance, reducing the liver, abolishing the edema and the nocturnal dyspnea. On March 2, 1937, coramin, 30 minims twice daily, was commenced. On March 7, the patient, no longer bedridden, resumed moderate activity which he increased gradually. Since June he has carried on his business comfortably, even to the point of being able to climb steps without distressing breathlessness.

CASE 2.—J. J. M., male aged 70 years, worked as a newspaperman until two years ago. At that time he suffered an acute coronary occlusion from which he recovered sufficiently to become ambulatory, but with such limited myocardial efficiency as to become dyspneic on the slightest effort. There had been no history of hypertension in earlier life. Physical examination showed a concentrically enlarged heart, diastolic pressure ranging above 100, and a diastolic murmur over the aortic area, probably due to a relative atherosclerotic aortic insufficiency. Fluoroscopy revealed a small amount of fluid in the right costophrenic sinus, both lungs were not well aerated and showed evidence of congestive failure, elongated, tortuous, and dilated aortic arch and thoracic aorta, markedly enlarged inflow and outflow tracts of both ventricles. The neck vessels were distended and pulsating, and the venous pressure was high. EKG showed low voltage and inverted T-waves. All treatment had proved ineffectual in restoring compensation; there was no longer a response to diuretics. Coramin given in 30 minim doses twice daily was commenced on March 18, 1937, and continued until May 27, 1937, with no improvement. Decompensation was growing more complete.

In summary it is obvious that there remained not a particle of reserve in Case 2. Myocardial fibrosis was probably so extensive, complete, and collateral, and coronary circulation so inadequate as to produce little or no response from any vasodilatory agent. In addition, central depression of circulation will mitigate materially against aiding peripheral circulation. Those cases failing to show any change under the influence of coramin medication fall under this category.

CONCLUSION

Out of a group of 17 patients suffering from the chronic coronary artery syndrome, observed clinically over a period of several months, and treated solely with coramin medication, 12 were considerably improved, kept free from symptoms, and maintained in reasonably complete economic restitution; 3 patients were slightly improved, and 2 unchanged. As an addition to the armamentarium of cardiac therapy, coramin is suggested in therapeutic doses of from 20 to 30 mimims twice daily, orally administered in fruit juices, in the type of patient described earlier in this paper.

REFERENCES

1. Cowan, J. H.: Observations on Coramine, Am. J. Med. Sc. **193**: 673, 1937.
2. Stoland, O. O., and Ginsberg, A. M.: Studies on Coronary Circulation, J. Pharmacol. & Exper. Therap. **60**: 396, 1937.

DETERMINATION AND QUANTITATIVE ESTIMATION OF THE DECOMPOSITION OF CHLOROPHYLL IN THE HUMAN BODY*

JOACHIM THEODOR BRUGSCH,† M.D., AND CHARLES SHEARD, PH.D.,
ROCHESTER, MINN.

VERY little clinical information is available regarding the decomposition of chlorophyll in human metabolism, although much is known concerning the chemical structure of the chlorophylls per se and their relation to the blood pigments and porphyrins III, as has been shown by H. Fischer,[4] Conant,[3] and Willstätter.[18]

Chlorophylls differ from hemins in that they have magnesium instead of iron in the porphin nucleus and have different side chains, particularly the isocyclic ring. By reason of this more complicated structure, chlorophylls and their higher derivatives show typical spectroscopic absorption bands which are very different, in both position and number, from the simpler spectra of heminogenous porphyrins. There is also the possibility of rotation and allomerization. By proper chemical procedures chlorophylls in vitro may be changed to etioporphyrin III. Through these transformations, the phylogenetic relation, which may exist between hemin and chlorophyll in the natural development of pigments, can be made clearer. Therefore, theoretically, simple porphyrins might occur as final products in the human metabolism of chlorophyll.

Experimentally, however, it has not been possible to prove that increased coproporphyrinuria was produced in the human body after feeding green vegetables, such as spinach, as has been shown by Brugsch,[2] and by Franke and Fikentscher.[9] On the other hand, phylloerythrin[13] and, more recently, pyrroporphyrin (Rothemund[15]) have been obtained as products of the decomposition of chlorophyll in beef bile. The alteration of chlorophylls in the enteric tract of ruminants has been investigated by H. Fischer and his collaborators[5, 6, 8] and by Rothemund.[14, 15] The hydrochloric acid in the stomach seems to have a pronounced effect upon the chlorophyll molecule in removing magnesium and possibly also phytol and methyl alcohol. Rothemund has found pheophytin *a*, pheopurpurin 18, and phylloerythrin in the gastric mucosa of cows. Further changes in the intestines are brought about by extensive reduction, decarboxylation, loss of rotation, and other modifications which culminate finally in the formation of phylloerythrin, a substance which is a very important final product of the decomposition of chlorophyll in bovine metabolism.

*From the Division of Biochemistry and Division of Physics and Biophysical Research, the Mayo Foundation, Rochester.
†Fellow in Biochemistry, the Mayo Foundation.
Received for publication, March 28, 1938.

There are a large number of steps in the decomposition between chlorophyll and phylloerythrin. Some of these compounds have been isolated and chemically analyzed by H. Fischer and his collaborators. The method of Willstätter is generally used to isolate the products of decomposition. This method is based on the fact that both porphyrins and products of the decomposition of chlorophyll are extracted from ether by specific concentrations of hydrochloric acid, depending on the basicity of the compounds. The concentration of hydrochloric acid necessary to extract them becomes proportionately lower as the change in decomposition of chlorophyll is increased. Willstätter defined the hydrochloric acid number as the percentage amount of this acid which will remove about two-thirds of the dissolved substance from an equal amount of ether. Using pure preparations, Willstätter obtained the data given in Table I.

TABLE I

THE RELATIONSHIP BETWEEN THE CONCENTRATIONS OF HYDROCHLORIC ACID AND THE EXTRACTION OF VARIOUS DECOMPOSITION PRODUCTS OF CHLOROPHYLL WITH ETHER

COMPOUND	HCl NUMBER	PERCENTAGE HCl ABLE TO EXTRACT PRACTICALLY TOTAL AMOUNT
Pheophytin *a*	29	32
Pheophytin *b*	35	--
Methylpheophorbide *a*	16	18
Methylpheophorbide *b*	21	23
Pheophorbide *a*	15	17
Pheophorbide *b*	19.5	22
Phytochlorin *e*	3	4-5
Phytorhodin *g*	9	11
Pyrroporphyrin	1.5	3
Rhodoporphyrin	3	4
Phylloporphyrin	0.75	1.5

METHODS

1. *Extraction.*—We have employed Willstätter's principle to obtain the decomposition compounds of chlorophyll which may exist in human feces. Ten grams of the amount of feces, collected in a period of twenty-four hours and, if possible, after feeding a meat-free diet, were treated with acetic acid and transferred into ether. After the removal of porphyrins, compounds of chlorophyll could be withdrawn from the ether by successive extractions with 10 per cent hydrochloric acid, 25 per cent hydrochloric acid, 37 per cent hydrochloric acid, using ether and hydrochloric acid in the proportion of about 3:1 by volume. The extraction is performed as follows:

Ten grams of the collected feces are ground in a mortar, and about 10 to 15 c.c. of glacial acetic acid is added. To this mixture, 75 to 100 c.c. of ether is added, and the mixture is again ground; the ether extract is then transferred to an Erlenmeyer flask. The remainder of the feces is ground repeatedly by adding a few cubic centimeters of glacial acetic acid and ether until the resultant extraction of ether appears practically colorless. All extracts of ether, together with the remainder of the ground feces, are shaken together in a machine for two hours. Then the mixture is passed through an ordinary paper filter and washed in a separatory funnel with distilled water

to remove the free acetic acid. The procedure is carried on until the wash water no longer turns blue litmus paper red.

The acid-free ether is shaken with small amounts of 5 per cent hydrochloric acid in order to remove the porphyrins. The shaking is repeated two to three times for fifteen minutes. The 5 per cent hydrochloric acid extract contains all the porphyrins but some protoporphyrin may remain in the ether, especially in cases in which the feces contain a large quantity of hemin compounds produced by the presence of blood or meat in the intestinal tract. This protoporphyrin may be obtained in the subsequent extraction with 10 per cent hydrochloric acid.

In the 5 per cent hydrochloric acid we have frequently found, by spectroscopic examination, derivatives of chlorophyll which are carried down along with the porphyrins. This is likely to occur in the presence of colloidal impurities, although the hydrochloric acid number of those compounds may be far higher than the concentration of hydrochloric acid used. In order to separate such chlorophyll compounds from porphyrins and to restore them to the ether extract, we have found it necessary to neutralize the 5 per cent hydrochloric acid extract with sodium acetate until Congo paper remains red. The porphyrins and chlorophyll compounds are then placed in a separatory funnel and are extracted by ether containing a few drops of glacial acetic acid. It is necessary to extract the neutralized solution of porphyrins and chlorophylls several times with ether, adding some glacial acetic acid to maintain an acid reaction in order to avoid the loss of chlorophyll derivatives. We have used the property of red fluorescence in ultraviolet light to determine the presence of small amounts of porphyrins or chlorophylls. Larger amounts of the compounds of the chlorophyll type may be determined with a pocket spectroscope, especially by observing the band in the red region. The ether-acetic acid extract must be washed with slightly alkaline (Na_2CO_3) distilled water and later with pure distilled water. The process is repeated until the acetic acid is removed from the ether. Subsequently the porphyrins may be removed by repeated shaking with 5 per cent hydrochloric acid. The decomposition products of chlorophyll which have higher hydrochloric acid numbers remain in the ether. The derivatives of chlorophyll which are in solution in ether are added to the ether extract, previously obtained, which contains the largest part of the chlorophyll compounds.

The foregoing separation of porphyrins from chlorophyll derivatives obtained from human bile and feces is adequate because, from our observations, phylloerythrin does not appear in any appreciable amount as an end product of chlorophyll metabolism. It would be difficult to separate phylloerythrin from other porphyrins, because the hydrochloric acid number is about eight (Brugsch and Keys[2]).

After the removal of porphyrins, the various chlorophyll derivatives in ether are extracted, in turn, with 10 per cent, 25 per cent, and 37 per cent hydrochloric acid. Each extraction is done three times with the same concentration of hydrochloric acid, the relation of the volume of ether to the volume of hydrochloric acid being about 3 to 1. In this manner three hydrochloric acid fractions are obtained finally: (1) 10 per cent, (2) 25 per cent, and

(3) 37 per cent hydrochloric acid; the remainder is (4) the ether fraction. Each fraction is examined spectroscopically for the presence of absorption bands characteristic of chlorophyll derivatives.

2. *Quantitative Estimations of Chlorophylls and Their Derivatives.*—Various methods may be used for the quantitative estimation of the chlorophylls or their derivatives which may be present in the four fractions.

A. Colorimetric determinations: the comparison of the intensity of the green color with the intensity of a standard solution of chlorophyll. This method can be used only for pure preparations (Willstätter).

B. Fluorescence determinations: the comparison of the intensity of the red fluorescence, in ultraviolet light, of an unknown amount of chlorophyll

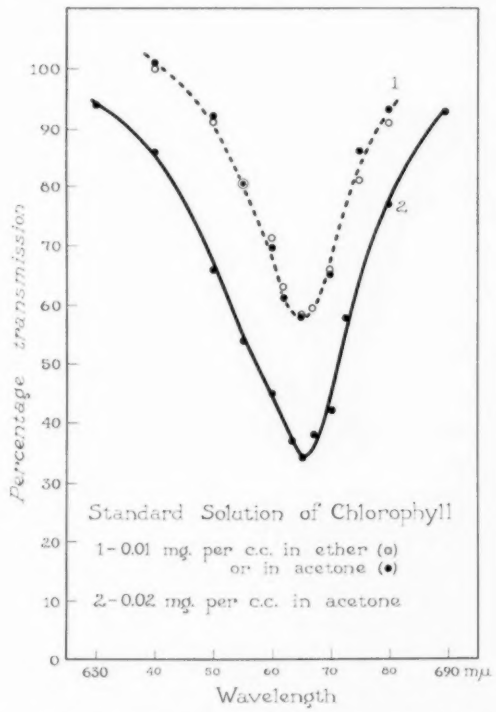

Fig. 1.—Curves of the spectrophotometric transmissions of solutions of crystallized preparations of chlorophyll in the region 630 to 690 millimicrons; *1*, 0.01 mg. per c.c. of ether or acetone; *2*, 0.02 mg. per c.c. of acetone.

derivatives with a standard fluorescent solution. One of us (Brugsch[1]) has used the fluorescence of coproporphyrin as a standard to determine the relative amounts of compounds of chlorophyll decomposition extracted with 25 per cent hydrochloric acid from human feces, biles, and urines. The difficulty in using this method is the fact that solutions of chlorophylls and also solutions of their derivatives, which have high hydrochloric acid numbers, lose some or all intensity of fluorescence when they pass into a colloidal state. It is not possible, therefore, to keep standard solutions of chlorophyll for several days without loss of fluorescence. Hence the quantitative estimation of fluorescence as a measure of the amount of chlorophyll compounds which may be present is not satisfactory.

C. Spectrophotometric determinations: the measurement of the percentage of transmission (or absorption) of light in the red region of the spectrum by solutions of chlorophylls and similar compounds and the comparison with the transmission (or absorption) of light in the same spectral region by a standard solution of chlorophyll in the same condition (acetic acid ether). We have used a direct reading spectrophotometer, the so-called Keuffel and Esser color analyzer. This apparatus and methods for the spectrophotometric determination of porphyrin were described by Sheard, Osterberg, and Goeckerman.[16]

We have used standard solutions containing 1 or 2 mg. per 100 c.c. of crystallized preparations of chlorophylls. These materials were obtained from the American Chlorophyll Co., and were mixtures of chlorophyll *a* and *b*. For each spectrophotometric determination we have prepared a fresh solution of chlorophyll in ether in the presence of acetic acid. A standard solution, which contains the same amount of chlorophyll in acetone, gives the same spectrophotometric reading as does a solution in ether. Fig. 1 shows the spectrophotometric readings obtained in the region of the red absorption band of three standard solutions of chlorophyll; two of them (curve 1) contain 1 mg. of chlorophyll per 100 c.c. of ether or acetone, and the third contains 2 mg. per 100 c.c. of acetone. The maximal absorption occurs at approximately 665 millimicrons. For control purposes, we also made simultaneously qualitative spectroscopic examinations of the solutions and determined the presence of other absorption bands of chlorophyll derivatives. This procedure enabled us to rule out the presence of compounds which have similar absorption bands.

It should be emphasized that preparations of chlorophyll are affected readily by acid and other vapors in the room, as well as by light. Hence it is essential that a new supply of crystalline chlorophyll be used for the preparation of a standard solution. When solutions are prepared in this manner, the spectrophotometric readings at the point of maximal absorption (that is, 665 millimicrons) are practically the same (that is, 57 and 60 per cent, respectively). We have found that crystalline chlorophyll, which has been kept for a few months, may show a shift of the maximal absorption to (approximately) 670 millimicrons.

We have taken 58 per cent as the average value of the spectrophotometric transmission of the standard 1 mg. per 100 c.c. of a solution of chlorophyll at 665 millimicrons. We have neutralized the free hydrochloric acid in the hydrochloric acid extracts of chlorophyll derivatives from human feces with sodium acetate and sodium hydroxide and then transferred into ether with acetic acid before obtaining the spectrophotometric readings on solutions containing unknown amounts of chlorophyll derivatives. In every instance we made the spectrophotometric determinations as soon as possible after the transfer into ether. The amount of pigment can be determined from the laws of Bouguer and Beer,

$$\frac{C_1}{C_2} = \frac{-\log I_1}{-\log I_2}$$

in which C_1 and C_2 are the respective concentrations, and I_1 and I_2 are the percentages of transmitted light at the maximal absorption value (that is, 660 to 670 millimicrons). The symbols C_1 and I_1 refer to the standard solution; in this case the wave length of maximal absorption is 665 millimicrons.

QUANTITATIVE ESTIMATIONS OF DECOMPOSITION OF CHLOROPHYLL SUBSEQUENT TO THE INGESTION OF A GENERAL TYPE OF DIET

Table II gives the values of the concentrations (per 100 gm. of feces) of the products of chlorophyll decomposition determined from the spectrophotometric readings and a substitution in the equation expressing the Bouguer-Beer law.

TABLE II

AMOUNTS OF CHLORPHYLL DERIVATIVES EXTRACTED BY VARIOUS CONCENTRATIONS OF HYDROCHLORIC ACID

CASE	10% HCl (MG. PER 100 GM. FECES)	25% HCl (MG. PER 100 GM. FECES)	37% HCl (MG. PER 100 GM. FECES)	ETHER (MG. PER 100 GM. FECES)
1	0.732	3.925	15.2	11.0
2	2.1	22.0	56.5	3.95
3	0.787	2.65	3.24	Negative
4	0.689	2.45	10.16	1.05
5	0.612	13.725	40.31	7.525
6	0.341	7.72	23.8	0.576
7	Traces	3.55	4.395	3.355
8	Traces	4.12	13.8	0.55
9	Negative	Negative	2.40	Traces
10	Negative	Negative	7.40	6.858
11	Negative	Negative	10.792	Negative
12	Negative	0.672	4.08	1.5
13	Negative	0.527	1.695	0.86
14	Negative	Negative	3.14	Negative
15	Negative	0.4575	41.31	?
16	Negative	3.4	6.27	4.877
17	Negative	Negative	0.63	0.48
18	Negative	Negative	4.14	3.05
19	Negative	1.89	4.21	2.27
20	Negative	0.816	4.725	2.16

1. *Pheophytin Fraction (37 Per Cent Hydrochloric Acid).*—The results obtained show that the largest part of decomposed chlorophylls found in human feces cannot be extracted from ether with 10 per cent or 25 per cent hydrochloric acid, but only with 37 per cent hydrochloric acid. Therefore, according to our methods and data, the largest part of the chlorophyll decomposition in the bowels has not progressed beyond its initial stage. This fraction may be called the pheophytin fraction, the largest part being pheophytin *a*. Curve 1 of Fig. 2 shows the spectrophotometric transmission of this fraction after neutralization of the hydrochloric acid with sodium hydroxide and the transfer into ether.

It is well known that pheophytins in concentrated hydrochloric acid are very unstable. Pheophytin is converted into pheophorbide readily by the action of hydrochloric acid. After standing for a few hours, pheophytins in 37 per cent hydrochloric acid are changed completely to pheophorbides. Hence we have allowed the compounds of the pheophytin fraction to remain in the 37 per cent hydrochloric acid for several hours in order to convert them into

phorbides. The phorbides are transferred into ether and again extracted; initially with 18 per cent hydrochloric acid to obtain the pheophorbide *a*, and later with 22 per cent in order to obtain the pheophorbide *b*. The 18 per cent hydrochloric acid contains the largest part of the pigments. This fraction is taken into ether and esterified with diazo methane (Fischer and Riedmair[7]). After standing twenty-four hours, pheophorbide is converted into chlorintrimethylester. This compound can be crystallized from acetone methyl alcohol and recrystallized out of ether methyl alcohol. The typical prismatic crystals have a melting point of 210° C.

2. *Phorbide Fraction (25 per cent hydrochloric acid).*—The 25 per cent hydrochloric acid fraction of the decomposition of human chlorophyll often contains compounds which, according to their hydrochloric acid numbers, are phorbides. Investigations of H. Fischer and Stadler[8] revealed some products of the decomposition of chlorophyll in feces of ruminants, especially of sheep. They found compounds such as pheophorbide *a*, pyrropheophorbide *a*, dihydro-

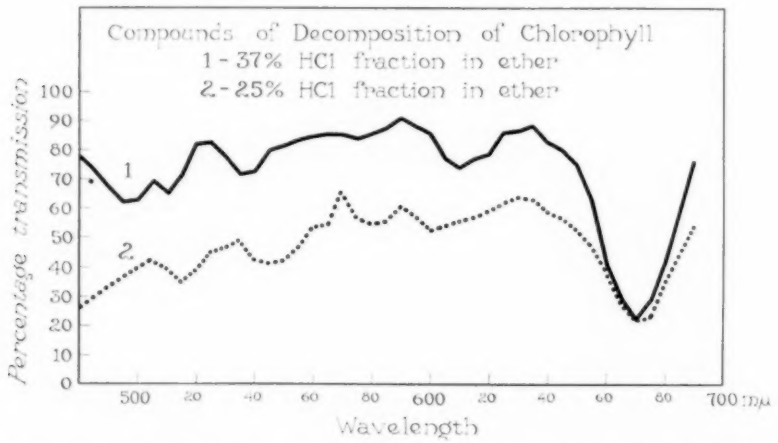

Fig. 2.—Curves of the spectral transmissions of the decomposition products of chlorophyll; *1*, 37 per cent hydrochloric acid fraction in ether; *2*, 25 per cent hydrochloric acid fraction in ether.

pheophorbide *a*, pyrropheophorbide *b*, or so-called probophorbides. We have not been able to determine whether these are the same compounds which occur in human chlorophyll metabolism, and, if so, which of them occur. The curve of spectrophotometric transmission of such a mixture obtained by extraction from ether with 25 per cent hydrochloric acid is shown in Fig. 2, curve 2. The phorbide fraction may be fairly large in relation to the total excretion of chlorophyll products but not in excess of (approximately) 40 per cent, and comprises a variable part of the total amount of such pigments in the feces (see Table II).

3. *The 10 Per Cent Hydrochloric Acid Fraction.*—The amount of decomposition products of chlorophylls obtained from the feces is the smallest in the 10 per cent hydrochloric acid fraction. These decomposition products are detectable only after large amounts of chlorophyll have been ingested. We were not able to prove the presence of phylloerythrin in human feces or bile. If we take the spectrophotometric transmission of this fraction in 10 per cent

hydrochloric acid fraction (after the removal of impurities through repeated transfers and extractions between ether and 10 per cent hydrochloric acid), we obtain curves (Fig. 3, curves 1 and 2) which show three or four bands in both human bile and feces (obtained by treating 10 liters with acetic acid ether) instead of the two bands typical of phylloerythrin in 10 per cent hydrochloric acid.

The spectrophotometric curves obtained from 10 per cent hydrochloric acid extracts of bile and feces differed markedly from the spectral transmission curve of phylloerythrin in 10 per cent hydrochloric acid in that they exhibit the strongest absorption band in the red region (Fig. 3). These data and curves, and similar results, show that, in the decomposition of chlorophylls in the human body, compounds appear in bile and feces which are extractable from ether with 10 per cent hydrochloric acid and which may be differentiated readily from phylloerythrin in 10 per cent hydrochloric acid by the presence

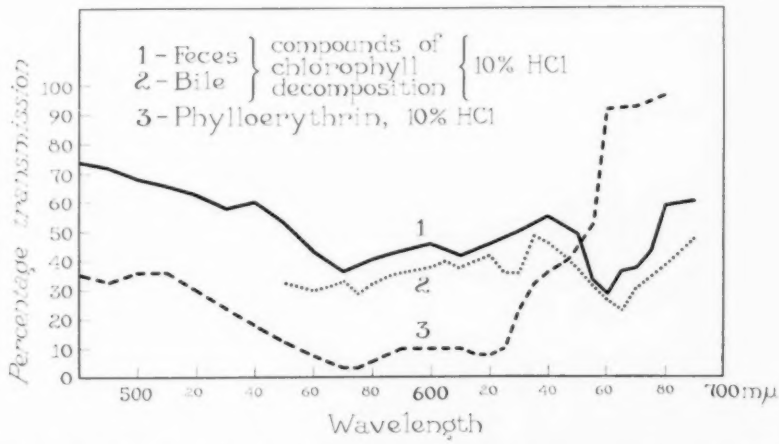

Fig. 3.—Curves of the spectral transmissions of the decomposition products of chlorophyll; *1*, 10 per cent hydrochloric acid extract of feces; *2*, 10 per cent hydrochloric acid extract of bile; curve *3* is the spectral transmission of phylloerythrin in 10 per cent hydrochloric acid.

of an absorption band in the red region of the spectrum. Furthermore, there is in ether a very noticeable difference spectroscopically, especially in the red region, between phylloerythrin and such compounds. The spectroscopic data, showing the presence and limits of the observed absorption bands, are: phylloerythrin in ether; I. 638 to 633 millimicrons, II. 597 to 579 millimicrons, III. 564 to 554 millimicrons, and IV. 528 to 513 millimicrons; in human decomposition of chlorophyll (10 per cent hydrochloric acid fraction); I. 673 to 656 millimicrons, II. 605 to 581 millimicrons, III. 565 to 554 millimicrons, and IV. 537 to 520 millimicrons.

4. *Chlorophyll Fraction (ether).*—The chlorophyll remaining in the ether after all the extractions with different concentrations of hydrochloric acid may be, in part, unchanged. On the other hand, some chlorophyll may have been changed but cannot be extracted by hydrochloric acid in the presence of impurities, especially fats and colloids in the ether.

QUANTITATIVE ESTIMATION OF DECOMPOSITION OF CHLOROPHYLL AFTER INTAKE OF CRYSTALLINE CHLOROPHYLL

In order to exclude the possibility of changes in chlorophyll produced by the preparation of the food prior to its ingestion, we gave several hundred milligrams of standard chlorophyll in capsules to persons with normal metabolism and on a chlorophyll-free diet. The data of Fig. 4 show the increase in the excretion of the various decomposition products under the administration of chlorophylls given in doses of 100 mg. a day for four days. We have made the same fractionations with 10 per cent hydrochloric acid, 25 per cent hydrochloric acid, and 37 per cent hydrochloric acid, as were used in the investigations following the ingestion of a general type of meal. The pheophytin fraction is largely predominant. In one instance we were able to recover about 43 per cent of the 400 mg. of chlorophyll ingested. In another case

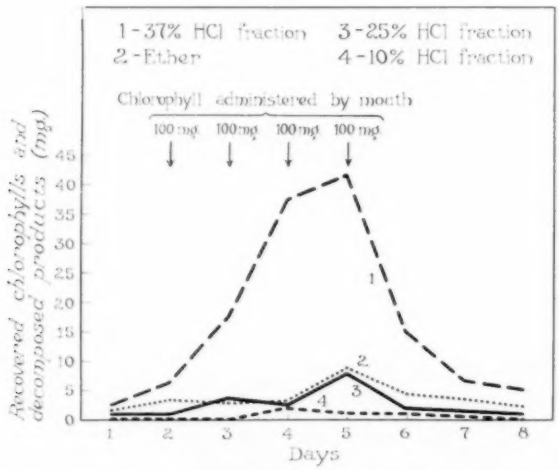

Fig. 4.—Curves showing the relationships between the intake of crystalline chorophyll and the recovery of chlorophyll and its products of decomposition; *1*, 37 per cent hydrochloric acid fraction; *2*, ether fraction; *3*, 25 per cent hydrochloric acid fraction, and *4*, 10 per cent hydrochloric acid fraction.

there was excreted 72.7 per cent of chlorophyll subsequent to the first administration of 100 mg. of material.

The question arises as to whether such a loss of chlorophyll compounds is due to difficulties of the methods of extraction, the presence of impurities, absorption, and so forth, or whether, in addition to the type of chlorophyll decomposition we have investigated, there are other ways and means for the body to break down those pigments.

It has been suggested that the chlorophylls are decomposed to porphyrins in the course of metabolism and appear in the urine. We have investigated quantitatively this possibility, over a considerable period of time, in different individuals with and without the administration of chlorophyll. In the twenty-four-hour specimens of urine we have found coproporphyrin with 113.6 gamma as the highest value and, three days after stopping the feeding of chlorophyll, there was still 80.6 gamma in the urine. On the other hand, the amounts of

porphyrins in the feces during the intake of 400 mg. of chlorophyll was 770 gamma as the highest value and only 48.6 gamma as its lowest value daily. It does not seem possible, therefore, that the normal process of chlorophyll decomposition in the human body should lead to large amounts of porphyrins.

There is another possibility of the breakdown of such compounds. H. Fischer and Stadler have mentioned the appearance of reduced "leuco" compounds which could be isolated as phylloerythrin by reoxidation of the compounds extracted from the wash water. We have not investigated the presence and the importance of such compounds. The question also arises as to whether there is any direct relationship between the presence of larger amounts of bilinoidal pigments in urine and feces after the ingestion of food rich in chlorophyll. This problem, in so far as we know, has not yet received any serious investigation.

SUMMARY

1. Methods are described by which it is possible to extract and to estimate spectrophotometrically the amounts of decomposed products of chlorophyll which are found in human bile and feces.

2. The spectrophotometric determinations of various chlorophyll compounds in ether are based on the intensity of transmission in the red region (approximately 665 millimicrons) as compared with the transmission in the same region, obtained with a standard solution of crystalline chlorophyll *a* and *b*.

3. The products of decomposition of chlorophyll were separated into four fractions: (1) 10 per cent hydrochloric acid, (2) 25 per cent hydrochloric acid (phorbides), (3) 37 per cent hydrochloric acid (pheophytins), and (4) ether (chlorophylls and others).

4. The pheophytin fraction was found to be the largest portion of the products of chlorophyll decomposition recovered from human feces.

5. The presence of pheophytin *a* was proved by the conversion into pheophorbide *a* and crystallization into chlorintrimethylester (melting point, 210° C.).

6. The 25 per cent fraction was found to contain phorbides.

7. Compounds soluble in the 10 per cent hydrochloric acid fraction were obtained from human feces and human bile; spectroscopic analyses showed them to be different from phylloerythrin, both in the feces and in bile. We were not able to prove the presence of phylloerythrin.

8. It is possible to obtain the same fractions of chlorophyll products in the feces after the ingestion of pure crystalline chlorophyll with a chlorophyll-free diet as under a general diet. The loss of the ingested crystalline chlorophyll may amount to as much as 57 per cent.

9. It is shown that the loss of chlorophyll is not due to the conversion into excreted porphyrins.

10. The possibilities concerning the fate of chlorophyll in human metabolism are discussed briefly.

REFERENCES

1. Brugsch, J.: Untersuchungen des quantitativen Porphyrinstoffwechsels beim gesunden und kranken Menschen, IV Mit. Ztschr. f. d. ges. exper. Med. B. **98**: 49, 1936; V Mit. Ztschr. f. d. ges. exper. Med. B. **98**: 57, 1936.
2. Brugsch, J., and Keys, A.: Quantitative Separation and Estimation of Biological Porphyrins in Minute Amounts, J. Biol. Chem. In Press.
3. Conant, J. B., and Bailey, E. M.: Studies in the Chlorophyll Series IX. a. a. in the same journal. J. Am. Chem. Soc. **55**: 795, 1933.
4. Fischer, H.: Chlorophyll, Chemical Rev. **20**: 41, 1937.
5. Fischer, H., and Hendschel, A.: Über den biologischen Abban der Chlorophylle, Ztschr. f. physiol. Chem. **198**: 33, 1931.
6. Fischer, H., and Hendschel, A.: Über Phyllobombyrin and Probophorbide, Ztschr. f. physiol. Chem. **206**: 255, 1932.
7. Fischer, H., and Riedmair, T.: Über die Aufspaltung von Chlorophyll "a" und seinen Derivaten durch Diazomethan, Liebig's Annalen **506**: 107, 1933.
8. Fischer, H., and Stadler: Gewinnung von Dihydrophäophorbid "a" und Pyrophäophorbid "b" aus Schafkot, Ztschr. f. physiol. Chem. **239**: 167, 1936.
9. Franke, K., and Fikentscher, R.: Die Bedeutung der quantitativen Porphyrin-bestimmung mit der Lumineszenzmessung für die Prüfung der Leberfunktion und für Ernährungsfragen, München. med. Wchnschr. **1**: 171, 1935.
10. Godinho, A. P.: Über das Auftreten eines roten Farbstoffes nach Einnahme von reinen Chlorophyll, Biochem. Ztschr. **155**: 90, 1925.
11. Hofstetter, Martha: Über den Nachweis von Chlorophyllabbanprodukten im Urin, Biochem. Ztschr. **155**: 80, 1925.
12. Kitahara, Yoshitaka: Über die Ausscheidung von Chlorophyll derivaten aus dem Urin III, Biochem. Ztschr. **155**: 97, 1925.
13. Marchlewski, L.: Über den Ursprung des Cholehämatins (Bilipurpurins), Ztschr. f. physiol. Chem. **45**: 466, 1905.
14. Rothemund, P.: Occurrence of Decomposition Products of Chlorophyll. II, J. Am. Chem. Soc. **56**: 2400, 1934.
15. Rothemund, P.: Occurrence of Decomposition Products of Chlorophyll. III. Isolation of Pyrroporphyrin From Beef Bile, J. Am. Chem. Soc. **57**: 2179, 1935.
16. Sheard, C., Osterberg, A. E., and Goeckerman, W. H.: The Spectrophotometric Determination of Hematoporphyrin in Urine, J. Lab. & Clin. Med. **15**: 162, 1929.
17. Willstätter, R., and Stoll, A.: Untersuchungen über Chlorophyll, Verl., 1931, Julius Springer.
18. Willstätter, R.: Die Blattfarbstoffe, Handb. d. biol. Arbeitsmeth., Abt. I, Teil 11, p. 1, 1936.

PRONTOSIL AND THE TREATMENT OF SPREADING PERITONITIS IN DOGS*

John O. Bower, M.D., and John C. Burns, M.D., Philadelphia, Pa., and Harold A. Mengle, M.D., Franklin, N. C.

IT IS NOT without some hesitancy that we present this report of our investigation of the effect of prontosil upon spreading peritonitis of appendiceal origin, experimentally produced in dogs. The early reports of the efficacy of sulfanilamide against coccal infection, especially streptococcal, meningococcal, and gonococcal, aroused a wave of enthusiasm which resulted in regrettable untoward effects and fatalities. Now that reports and warnings of various ill effects have been published, perhaps the medical profession will view favorable conclusions from recent experimental work more deliberately than heretofore.

*From the Department of Research Surgery, Temple University School of Medicine, Philadelphia, Pa.

Received for publication, March 28, 1938.

While our report deals with prontosil—disodium 4-sulfamido-phenyl-2-azo-7-acetylamino-1-hydroxynaphthalene 3-6-disulfonate*—it is well known that this substance is closely related chemically to sulfanilamide and in the body is converted into and depends upon the action of that substance for its effects. For obvious reasons, therefore, we merely present the results we obtained in experimental animals without further comment. We have not used it for the treatment of spreading peritonitis in man in a sufficient number of cases to make a report.

We have already reported an extensive study of the bacteriology of appendiceal peritonitis in both man and dog.[1] In brief, this study shows the predominating organisms in man to be *B. coli* (66 per cent of cases) and various strains of streptococcus (39 per cent of cases). In the dog, the incidence of *B. coli* was much lower (approximately 27 per cent), while that of the streptococcus remained about the same (32 per cent of cases). In both man and dog, *Cl. welchii* was present as the predominating anaerobic organism (60 per cent and 63 per cent, respectively). The high incidence of streptococci (of various strains) and *Cl. welchii* prompted this investigation.

The method of induction of spreading peritonitis in dogs and its treatment with horse serum, perfringens antitoxin, and lyophilized convalescent serum, have been previously reported.[2] The reasons for selecting dogs for this experimental work are of sufficient importance, however, to warrant repetition:

1. Dogs are always available for experimental work.

2. While the appendix of the dog is very much larger than that of the human being, especially when compared to the size of the intestine, it has a similar structure, and its base and mesentery can be easily ligated and gangrene readily induced.

3. The peritoneum of the dog is more resistant to infection than that of any other animal having an appendix. This is a decided advantage because the gradual development of the acute process makes possible accurate observation of gross changes and facilitates removal of tissue and exudate for histologic and bacteriologic examinations.

4. The constitutional resection of the dog following an induced gangrene of the appendix is similar to that observed in the human being when the appendix is acutely inflamed. Temperature is moderately elevated; the heart rate is moderately increased; the dog vomits and remains in one position unless disturbed. Examination shows a neutrophilic leucocytosis, and the blood culture is invariably negative for microorganism. When rupture occurs, the temperature and pulse are greatly increased, the tongue becomes dry, the eyes lusterless, and the abdomen moderately distended.

5. In addition, the complications of mechanical ileus and metastatic abscesses do not develop frequently, so that the symptoms and signs accompanying these hazards do not have to be dealt with.

Following the induction of gangrene of the appendix, in approximately 50 per cent of the dogs, a small localized abscess forms, and they recover without any treatment. The intraperitoneal changes which accompany recovery closely

*The prontosil for this investigation was furnished by the Winthrop Chemical Co.

resemble those observed in the human being. A certain percentage, however, will develop peritonitis and die. This percentage is increased by the administration of laxatives, just as in the case of man. The time of administration, kind and quantity of laxative administered, influence the mortality decidedly.

TECHNIQUE

1. *Selection of Animals.*—Dogs were selected at random, except that the control and treated dogs were of approximate size and weight. Pregnant dogs and those weighing less than 5 kg. were not used. Occasionally dogs were available that had been operated upon previously; but those that had abdominal operations and had been returned to stock within thirty days were not used. No effort was made to prepare the dogs for operation, except that they were not fed that morning.

2. *Operative Technique.*—All the operations were carried out under ether anesthesia. The abdomen was shaved, scrubbed with ether, and painted with 3.5 per cent tincture of iodine. Using aseptic technique, a right rectus incision, about 10 cm. long, was made, the appendix located, drawn up, dissected free of its mesenteric attachment, and all bleeding vessels clamped and separately ligated with fine silk. A double ligature of silk was then tied tightly around the appendix as close to its base as possible. After inspection to insure complete hemostasis, the abdominal contents were replaced and the wound closed in layers, using silk throughout and re-enforcing the fascia with interrupted sutures. Drains were not used. During the operation, precautions were taken to avoid the escape of blood into the peritoneal cavity. No dressings were applied.

3. *Administration of Laxatives.*—Early in our study of appendiceal peritonitis we found that by giving 30 c.c. of castor oil to the average dog *twenty-four hours after operation*, a spreading peritonitis could be induced in about 70 per cent of the cases. The mortality of this particular series was 68.5 per cent; 91.9 per cent of the deaths were due to spreading peritonitis. We then gave 30 c.c. of castor oil to a group of animals *directly after operation.* The mortality rose to 78.3 per cent, and 94.5 per cent died of spreading peritonitis. An average dose of 54 c.c. of castor oil was then given to a third group of dogs *immediately after operation;* the mortality rose to 91.7 per cent, and 100 per cent died of spreading peritonitis.[2] These experiments proved that the time of administration of laxatives and the quantity of laxative administered influence the outcome. Every animal that received 2 ounces of castor oil the day of operation, or twenty-four hours following the operation, died of spreading peritonitis.

4. *Postoperative Care.*—The animals were placed in metabolic cages and allowed to recover before being returned to the animal house. Food and water were placed before them. They were disturbed only when brought to the operating room for the administration of prontosil. They were returned to the animal rooms immediately after this procedure, with as little handling as possible. Special care was taken to avoid squeezing or massaging their abdomens.

Post-mortem examinations were carried out in all instances as soon as possible. If post-mortems were not done to determine the cause of death, if an

animal died of pneumonia or other complications within ten days, or if at subsequent operation the appendix was found to be intact, showing that the ligature had slipped, then the record was discarded.

RESULTS OF THE TREATMENT WITH PRONTOSIL OF INDUCED SPREADING PERITONITIS IN DOGS

Twelve dogs were used as a control group for comparison with the treatment group. The average weight of these control dogs was 11.6 kg. The appendix was ligated at its base, and the mesentery was stripped free and

TABLE I

PRONTOSIL

Animals That Recovered

DOG NO.	WEIGHT KG.	PRONTOSIL TOTAL DOSE C.C.	AVG. DOSE C.C. IN 8 HR.	NO. LIVED	SUBSEQUENT OPERATION APPENDIX ABSORBED
96	11	55	11	1	1
111	9	45	9	1	1
570	15	75	15	1	1
760	10	50	10	1	1
763	6.6	32.5	6.5	1	1
765	10	50	10	1	1
777	11.5	51.75	8.6	1	1
784	13	65	13	1	1
786	18	90	18	1	1
788	14	70	14	1	1
791	15	60	15	1	1
	12.1	58.6	11.8	11	100 per cent

PRONTOSIL

Animals That Died

DOG NO.	WEIGHT KG.	PRONTOSIL TOTAL DOSE C.C.	AVG. DOSE C.C. IN 8 HR.	NO. DIED	AVG. HR. LIVED AFTER OP.	PATHOLOGY SPREADING PERITONITIS
94	10	30	10	1	36	1
100	6	30	6	1	48	1
101	10	50	10	1	120	1
102	5	25	5	1	48	1
110	8	24	8	1	24	1
146	14	56	14	1	48	1
754	15	75	15	1	226	1
759	9	40.5	6.7	1	80	1
769	9.3	37.5	7.5	1	48	1
787	13	65	13	1	132	1
793	11	49.5	8.2	1	72	1
	10.3	43.9	9.4	11	80.18	100 per cent

ligated separately. An average dose of 54 c.c. of castor oil was given immediately after operation. The mortality was 91.7 per cent; 100 per cent died of spreading peritonitis, and the average number of hours those that died survived the operation was 66. In the treatment group were 22 dogs. The average weight was 11.03 kg.; the average weight of those that lived was 12.1 kg., and of those that died, 10.03 kg. With two exceptions, the dose of castor oil was 60 c.c. Two dogs in the treatment group received only 45 c.c. of castor oil,

instead of 60 c.c. One of these dogs lived and one died, No. 763 and No. 793, respectively. In the treatment group, those that recovered or died received the same amounts of castor oil.

Dose and Method of Administration of Prontosil.—Each dog was given 1 c.c. of prontosil per kilogram of body weight every eight hours, day and night. It was given intramuscularly, because our previous experience with perfringens antitoxin had shown that the intramuscular injection was more effective.[2] Each of the 22 dogs received an average dose of 51.2 c.c. total of prontosil. Those that recovered received an average total dose of 58.6 c.c., and those that died, 43.9 c.c. The average individual dose of those that lived was 11.8 c.c.; of those that died, 9.4 c.c. The diminished total dose of those that died is due to the fact that death interrupted further treatment. The animals that died survived the operation an average of 80.1 hours. The mortality of this group was 50 per cent; 11 dogs lived, 11 died. In those that lived, subsequent operation showed that the appendix was completely absorbed in every instance. Of those that died, 100 per cent died of spreading peritonitis. Table I shows the weight, total dose of prontosil, and the outcome of each individual dog.

SUMMARY

Twenty-two dogs, average weight 11.1 kg., were operated upon under ether anesthesia. Gangrene of the appendix was induced by ligating the blood vessels in the mesentery and the base of the appendix.

Spreading peritonitis was induced by the administration of 58.6 c.c. (average dose) of castor oil immediately after operation.

One cubic centimeter of prontosil per kilogram of body weight was injected intramuscularly every eight hours, day and night, until the animal had either died or shown sufficient improvement to indicate he would recover. The mortality of this group was 50 per cent.

A control group of 12 animals, average weight 11.6 kilograms, was operated upon as described, given 54 c.c. (average dose) of castor oil immediately after operation, but received no treatment. The mortality of this group was 91.7 per cent.

REFERENCES

1. Bower, John O., Burns, John C., and Mengle, H. A.: The Bacteriology of Spreading Peritonitis Complicating Acute Perforative Appendicitis, A Clinical and Experimental Study, Surgery 3: 645, 1938.
2. Bower, John O., Burns, John C., and Mengle, H. A.: Induced Spreading Peritonitis Complicating Acute Perforative Appendicitis, Surg. Gynec. & Obst. 66: 947, 1938.

THE ACTION OF CHEMICAL AND PHYSICAL AGENTS ON CLOSTRIDIUM WELCHII AND ITS TOXIN*

FRANCIS E. COLIEN, PH.D., OMAHA, NEB.

SINCE the early investigations of Minck (1896), considerable work has been reported on the effects of x-rays and ultraviolet light on microorganisms. Moore and Kersten (1936) prepared a nontoxic vaccine of *Shigella dysenteriae* (Shiga) by irradiation of saline suspensions of the organisms with soft x-rays. In 1937, by the same method, they inactivated encephalitis virus (St. Louis type). Blank and Kersten (1935) showed that a modified extract agar could be so altered by the action of soft x-rays that it would no longer support the growth of *Bacillus subtilis*.

This preliminary report deals with a study of the effect of x-rays on the toxin and organisms of *Clostridium welchii*. Kelly (1933) and Kelly and Dowell (1936) reported on the use of x-rays in the treatment of gas gangrene. In a later paper, Kelly and others (1937) were able to show some helpful effects by treating guinea pigs injected with *Clostridium welchii*, or its toxin, with x-rays. By irradiation of these animals some therapeutic action was shown. In some of the experiments, over 50 per cent of the animals treated lived while the controls died in a few days with typical gas gangrene. In other cases, the x-ray treatment seemed to increase the time, by several hours or days, between the inoculation and death of the animals. Irradiation also destroyed or decreased the gas in the tissues in a number of the animals.

APPARATUS AND TECHNIQUE

Two cultures of *Clostridium welchii* (No. 88 and 88 a) isolated from fatal cases of gas gangrene were used in this study. The cultures were purified by the usual anaerobic methods.

The toxin was produced by growing the organisms in beef infusion broth, pH 7.8, containing 0.1 per cent dextrose and sterile guinea pig tissue. The cultures were incubated for twenty to twenty-four hours at 37° C, centrifuged and filtered through a Berkefeld N filter. Six-tenths of a cubic centimeter of this filtrate killed 350 gm. pigeons in eight to ten hours.

Standard x-ray equipment and tubes were used. The tubes were operated at a voltage of 90 kv. and a current of 5 Ma. The material was irradiated for eight minutes at a distance of 30 cm. from the copper target. A 1 mg. aluminum filter and a cone were used throughout the work.

*From the Department of Bacteriology and Preventive Medicine, Creighton University School of Medicine, Omaha.

Received for publication, April 6, 1938

EXPERIMENTAL

In these experiments we were primarily interested in studying the effects of x-rays and biochemical substances on *Clostridium welchii* and its toxin. The material to be irradiated was placed in long slender glass tubes (1.5 by 20 cm.) and sealed with a rubber cap. The thickness and type of glass used in these tubes was taken into consideration in determining the radiation, which was equivalent to that used in treating patients with gas gangrene and similar infections.

In order to simulate conditions found in gas gangrene infections, the following substances were irradiated: 6 c.c. of a toxic filtrate with sterile guinea pig tissue, 6 c.c. of a toxic filtrate, 6 c.c. of an eighteen-hour culture of *Clostridium welchii* in beef infusion broth in the presence of sterile tissue, and a toxin leucocyte mixture consisting of 1.5 c.c. of a 0.5 per cent suspension of human cells in saline and 1.5 c.c. of toxin. The materials were prepared in duplicate so that one tube could be treated with x-rays while the other served as a control. The tubes containing the various substances were kept at room temperature for four hours before the first x-ray treatment. They were irradiated for a period of eight minutes every twenty-four hours for five successive days, and kept at room temperature throughout the experiment. Four hours after the first, second, and fifth treatments, the toxicity of the substances was tested on pigeons. The viability of the organisms was also tested by subculture in beef heart medium. The results of these tests are summarized in Table I.

TABLE I

ACTION OF X-RAYS ON *Cl. welchii* AND ITS TOXIN

MATERIAL INJECTED INTO PIGEONS*	TIME OF DEATH AFTER FIRST TREATMENT	CONTROL	TIME OF DEATH AFTER SECOND TREATMENT	CONTROL	TIME OF DEATH AFTER FIFTH TREATMENT	CONTROL
Culture 0.6 c.c.	6.5	6	7	6	7	6.5
	8.5	7	10.5	8.5	8	7
Toxin and tissue 0.6 c.c.	20	18	22	17	Lived	Lived
					Lived	Lived
Toxin 0.6 c.c.	8	8	8.5	8	7.5	8
					8	9
Toxin and leucocytes 1.2 c.c.	9	8	9.5	9		
	14	9	14	13.5		

*Weight of pigeons 350 to 400 gm.

Similar results were obtained when the above experiment was repeated using a more toxic filtrate given in 0.3 c.c. dosage.

No marked decrease in toxicity or viability was observed after irradiation of toxin, toxin-leucocyte suspension, or cultures of *Clostridium welchii*. Toxin in the presence of sterile tissue, treated or not treated with x-rays, gradually loses its toxicity for pigeons. This loss of toxicity suggests that tissue may absorb toxin or contains some definite substances which when released from tissue, either by means of x-rays or by disintegration, destroy toxin. In order to learn more about this action, an attempt was made to study the effect on toxin of various substances that are present in tissue and that might be released by x-rays.

A 3 per cent solution of hydrogen peroxide and 2 per cent solutions of cystine and glutathione were mixed with toxin, allowed to stand at room temperature for six hours, and injected intramuscularly into pigeons weighing 350 to 400 gm. In one experiment mixtures of 0.6 c.c. of toxin and 1 c.c. each of hydrogen peroxide, cystine, and glutathione were injected. The pigeons that received the hydrogen peroxide and glutathione lived; those that received the cystine died in forty hours, and the controls died in eight hours. In another experiment pigeons were injected with mixtures of 0.8 c.c. of toxin and 0.2 c.c. of hydrogen peroxide, and 0.5 c.c. each of cystine and glutathione. The pigeons receiving the hydrogen peroxide lived, those receiving the cystine died in thirteen hours, the glutathione in forty hours and the controls in seven hours. Three pigeons were injected with each of the toxin-chemical mixtures used in the above experiments. The results seem to indicate that certain chemical substances may play some part in detoxifying or altering toxin.

An attempt was made to determine the suitability of rabbits in studying the effects of x-rays on gas gangrene. Mature animals, weighing 3,500 to 4,000 gm., and young animals of 800 to 900 gm. were used. No definite lesions or gas were produced in 4 mature rabbits injected intramuscularly with 0.5 c.c. of a twenty-four-hour culture of *Clostridium welchii*. In a series of 8 young rabbits (four to five weeks old) a small perforating necrotic lesion appeared in five days and gradually increased in size until the entire muscles of the thigh were involved. The muscle tissue became soft, with some evidence of suppuration, and was gradually digested until the femur was exposed. There was no evidence of gas at any time.

DISCUSSION

Many investigators have found injurious or lethal effects produced on bacteria by x-rays, while others have failed to detect such effects. The results observed may be due to differences in the experimental conditions or to the nature of the organisms used. Lacassagne (1928) suggested that bacterial suspensions may show extreme degrees of variation in resistance to radiation due to some physiologic peculiarity. In our experiments we were not able to show that x-rays destroyed either the toxin or organisms, with an amount of irradiation equivalent to that used in treating infections in man. There is the possibility that we were attempting to destroy too great an amount of toxin in proportion to the dosage of x-rays used, or that toxin is not destroyed by x-rays. Further experiments are being conducted, using smaller amounts of toxin.

There are substances present in tissue, such as cytochrome, glutathione, and cystine (present through oxidation of glutathione), which may be liberated or stimulated to increased activity by x-rays. These substances may destroy or alter toxin, possibly through an oxidation process. The catalase present in tissue may be modified or destroyed by irradiation, and thereby permit hydrogen peroxide to accumulate, with the result that the toxin is altered. Our evidence as yet is only fragmentary, but may help to explain the non-specific effects of x-rays in the treatment of bacterial infections.

Desjardins (1935) believed that the destruction of infiltrating leucocytes, with the subsequent liberation of antibodies and other protective substances, plays a part in the rapid action of x-rays in curing certain acute and chronic infections. Although our preliminary experiments are not conclusive, we were able to show that leucocytes had little or no effect on toxin in the absence of living tissue.

Cornell (1925) produced chronic infections in mature rabbits with *Clostridium welchii*. Rabbits injected subcutaneously showed no marked symptoms, local reactions, or the presence of gas. We obtained similar results in mature animals, but were able to produce extensive local lesions in young rabbits; no gas was detected. There is a possibility that young rabbits may be of value in studying acute *Clostridium welchii* infections.

SUMMARY

1. No marked decrease in toxicity or viability was observed after irradiating toxin or cultures of *Clostridium welchii* with soft x-rays.

2. Considerable amounts of toxin may be absorbed or altered in the presence of sterile tissue.

3. Certain substances, such as glutathione, hydrogen peroxide, and to a slight extent cystine, may play some part in detoxifying or altering toxin in tissue.

4. Extensive local lesions were produced in young rabbits with pure cultures of *Clostridium welchii*.

The author acknowledges his appreciation to Dr. J. F. Kelly and Dr. D. A. Dowell, of the Department of Radiology, for their assistance in x-raying the materials.

REFERENCES

1. Minck, F.: Zur Frage über die Einwirkung der Röntgen'schen Strahlen auf Bacterien und ihre eventuelle therapeutische Verwendbarkeit, München med. Wchnschr. 43: 100, 1896.
2. Moore, H. N., and Kersten, H.: A Preliminary Note on the Preparation of Non-Toxic Shiga Dysentery Vaccines by Irraddiation With Soft X-rays, J. Bact. **31**: 581, 1936.
3. Moore, H. N., and Kersten, H.: The Inactivation of Encephalitis Virus (St. Louis Type) by Means of Soft X-rays, J. Bact. **33**: 625, 1937.
4. Blank, I. H., and Kersten, H.: The Inhibition of Growth of Bacillus Subtilis on a Modified Extract Agar by X-Radiation of the Mediums, J. Bact. **30**: 21, 1935.
5. Kelly, J. F.: The X-ray as an Aid in the Treatment of Gas Gangrene, Radiology 20: 296, 1933.
6. Kelly, J. F., and Dowell, D. A.: Present Status of the X-rays as an Aid in the Treatment of Gas Gangrene, J. A. M. A. **107**: 1114, 1936.
7. Kelly, J. F., Dowell, D. A., Russum, C. B., and Colien, F. E.: X-ray Treatment in Cases of Gas Gangrene, Radiology. In Press.
8. Lacassagne, A.: Action des rayons K de l'aluminium sur quelques Microbes, Compt. rend. Acad. d. sc. **186**: 1316, 1928.
9. Desjardins, A. U.: Radiotherapy (Roentgen Rays; Radium), J. A. M. A. **105**: 2153, 1935.
10. Cornell, B. S.: A Chronic Infection With B. Welchii, J. Infect. Dis. **36**: 425, 1925.

THE ROLE OF THE UPPER GASTROINTESTINAL TRACT IN THE ETIOLOGY OF PERNICIOUS ANEMIA*

An Experimental Study in Dogs

W. H. Bachrach, B.S., and S. J. Fogelson, M.D., Chicago, Ill.

AFTER reviewing the literature on the utilization of normal laboratory animals in assaying antipernicious anemia substances, we[1] concluded that no animal has as yet been found to exhibit a uniform or characteristic reaction to such substances. We then decided that the ideal laboratory assay for determining liver potency would be attained only after production of experimental pernicious anemia. The objective of this report is to present our experimental results in attempting to produce this disease in dogs.

RATIONALE FOR EXPERIMENTAL PROCEDURE

Castle and his associates have developed the most widely accepted theory on the etiology of pernicious anemia. They have shown that in the patient with pernicious anemia there is a deficiency or absence of some factor essential to the formation of the thermostabile liver substance. This factor, apparently of enzymic nature, was first believed to originate only in the stomach. Other investigators have shown it to be also present in the duodenum. These observations have explained the failure to produce pernicious anemia, not only after total gastrectomy, but also after exhaustion of the blood-forming precursors in these animals by prolonged bleeding.[6]

Dog liver has been shown to be effective in pernicious anemia,[10-13] but is only one-fifth to one-fourth as potent as hog liver. Dog stomach[2] and dog gastric juice[8] are apparently inert. These findings suggest that the intrinsic factor is either not present in the dog or is produced elsewhere in the intestinal tract. If the dog has no intrinsic gastric factor, there must be some other way of synthesizing the thermostabile liver substance, because we have observed dogs on liver-free diets for over two years without the development of pernicious anemia. Another possibility is that the amount of intrinsic factor in the dog's stomach is small and of relative unimportance as compared to the quantity present elsewhere in the upper intestinal tract. This conclusion was the basis for our experiments.

METHODS

Mongrel dogs of both sexes were kept preoperatively on liberal general diets for two or three weeks. Under ether anesthetic the jejunum was tran-

*From the Departments of Physiology and Surgery, Northwestern University Medical School, Chicago.

Received for publication, April 13, 1938.

sected 30 to 40 cm. distal to the ligament of Treitz. The distal opening was then immediately closed. The dissection was carried oralward until 30 cm. of jejunum, the entire duodenum, and the distal seven-eighths of the stomach

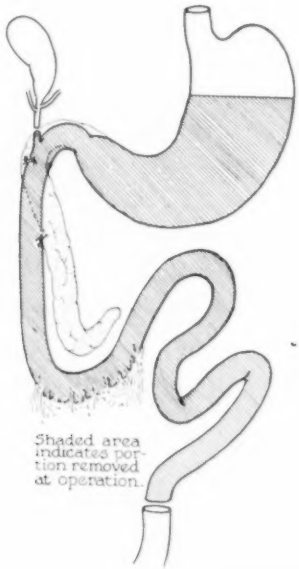

Fig. 1.

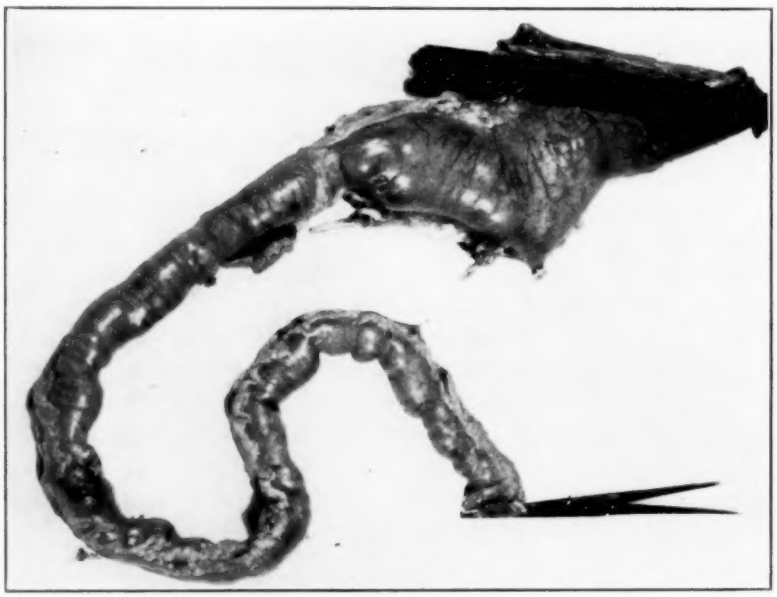

Fig. 2.—Extent of gastrointestinal tract removed.

were removed, care being taken to avoid trauma to the pancreas or impairment of its blood supply. The extent of the dissection and the portion of the alimentary tract removed are shown in Fig. 1. In some of the dogs more of

the stomach was removed than is indicated in Fig. 2. The lesser pancreatic duct was divided between ligatures. The major pancreatic duct and the common bile duct were then isolated and dissected free of the duodenum. Gastrointestinal continuity was re-established by an end-to-side anastomosis of the end of the stomach to the side of the jejunum, leaving a freely mobile afferent loop into which the common bile and major pancreatic ducts were anastomosed, both having already been dissected away from the duodenum (Fig. 3). The dogs were then placed upon a liberal diet without liver. They have been exercised daily outdoors and are very active.

Previous experiments have shown that normal values for red blood cells, hemoglobin, and hematocrit varied considerably, not only in different dogs, but in the same animal at different times. We therefore established our normal values for dogs in our laboratory by repeated determinations on 31 normal dogs, kept for several weeks on a stock diet (Table I). The blood for these determinations was obtained by venepuncture and collected in heparin tubes. Red blood cell counts were determined by standardized pipettes;

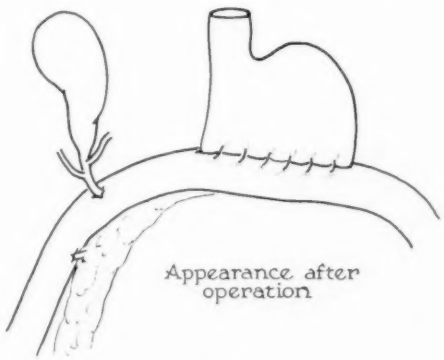

Fig. 3.

hemoglobin by the Sahli method; cell volume was measured in thick-walled hematocrit tubes of 1 mm. bore, after centrifugation at 1,000 and 2,000 r.p.m. for five minutes each.[11] For convenience in the calculation of our indices, we took red blood cells of 7,000,000, hemoglobin 14.1 gm. and hematocrit of 50, to represent 100 per cent.

The secretion of hydrochloric acid was estimated by withdrawing a sample of gastric contents at one-half hour intervals, following introduction of 120 c.c. of 7 per cent alcohol by stomach tube. The gastric response following the injection of 1 mg. of histamine hydrochloride subcutaneously was measured in the same way. Gastric emptying time was studied under the fluoroscope after the dogs had been given a meal containing one pound of commercial dog food, 100 c.c. of water, and 50 gm. barium sulfate. Roentgenograms were made at frequent intervals to determine any increase in size of the stomach.

RESULTS

Within a few months postoperative the 7 dogs comprising this series were indistinguishable from normal animals.

TABLE I

RED BLOOD CELL, HEMOGLOBIN, AND HEMATOCRIT DETERMINATIONS ON 31 NORMAL DOGS

RED BLOOD CELLS MILLION	HEMOGLOBIN* PER CENT	HEMATOCRIT
5.70	89	45
5.85	91	47
6.15	100	48
6.20	101	45
6.27	92	45
6.29	102	47
6.17	97	44
6.49	85	41
6.50	99	47
6.53	104	50
6.70	92	46
6.71	98	48
6.74	113	53
6.80	101	47
6.83	95	49
6.87	109	49
6.97	105	46
6.98	106	50
7.05	99	50
7.05	109	53
7.07	97	47
7.10	103	54
7.13	108	51
7.18	108	56
7.43	100	49
7.71	114	56
7.74	99	48
7.91	103	51
8.14	107	55
8.26	97	48
9.34	117	54
Average 6.97	101.3	49

*Hemoglobin 100 per cent = 14.1 gm.

Blood Pictures.—At no time did any of these 7 animals show a blood picture even suggestive of pernicious anemia. Detailed hematologic data on each dog would serve no purpose. They all exhibited a postoperative secondary anemia from which all but one recovered. This one persisting case of anemia, which is of eighteen months' duration, can hardly be attributed to reduction in gastric acidity, because gastric function in 4 of the remaining animals has been equally impaired. Table II summarizes the blood picture of each dog. The blood values in all these animals apparently showed greater fluctuations than customary in animals subjected to other major abdominal interference. Perhaps the instability of the blood regulatory mechanism may be secondary to the extensive surgical intervention. Reticulocyte counts and blood smears made at various intervals were essentially normal.

Biopsies of femoral bone marrow shaft showed nothing microscopically other than perhaps a moderate activity which was relatively insignificant as compared with the hyperplasia seen in gastrectomized dogs, but not suggestive to the slightest degree of pernicious anemia. The femoral bone marrow of Dog 123, sacrificed two years postoperative, appeared grossly almost entirely fatty.

TABLE II

SUMMARY OF POSTOPERATIVE RED BLOOD CELLS, HEMOGLOBIN, AND HEMATOCRIT ON DOGS IN THIS SERIES

DOG	RED BLOOD CELLS MILLIONS	HEMOGLOBIN GRAMS	HEMATOCRIT
90	7.16	14.41	51
106	6.93	12.90	47
115	5.88	12.01	41
120	6.17	14.12	47
123	6.98	13.49	47
127	7.73	14.12	47
143	7.35	15.26	46

Gastrointestinal Studies.—Fluoroscopic and roentgenologic studies of the residual stomach segment showed a gradual increase to practically normal size; despite this 3 of the 7 animals in this series remained almost entirely achlorhydric to alcohol and histamine stimulation, as determined by our method of fractional analysis. Our recent study of 28 normal dogs showed an average of 40 clinical units of free acid for the peak secretion to alcohol and 90 clinical units to histamine. In over 300 fractional gastric analyses performed on normal dogs, we have never found one refractory to stimulation by alcohol. The three dogs, Nos. 120, 127, and 123, gave no free acid response either to alcohol or to the subcutaneous injections of 1 mg. histamine hydrochloride, which justifies our assumption that they are unable to secrete practically any acid. Table III compares the acid secretion of the 7 dogs to the average in 28 normal animals. Perhaps by Necheles and Scheman's technique,[7] small amounts of free hydrochloric acid might have been demonstrated by constant aspiration.

TABLE III

POSTOPERATIVE GASTRIC ACIDITY VALUES CONTRASTED TO NORMAL

TIME HOURS	NORMAL	DOG 90	DOG 106	DOG 115	DOG 120	DOG 123	DOG 127	DOG 143
Response to Alcohol								
		CLINICAL UNITS FREE ACID						
0.5	38	0	55.0	0	0	0	0	0
1.0	40	17.5	10.0	0	0	0	0	0
1.5	25	0	0	0	0	0	0	0
2.0	13	0	0	0	0	0	0	0
Response to Histamine								
0.5	90	35.0	27.5	0	0	20.0	0	0
1.0	87	32.5	42.5	0	0	0	0	0
1.5	52	0	55.0	0	0	0	0	0
2.0	24	0	0	0	0	0	0	0

The emptying time of a standard meal was proportionate to the size of the stomach. Those dogs with the greatest hypertrophy of the gastric remnant, Nos. 90, 106, 115, and 120, had an emptying time which approached normal.

The following are protocols of two dogs representative of the group:

Dog No. 90.—Operated upon Sept. 20, 1935; weight 39 pounds. Following operation the red blood count was 4.71, Hb. 11.3 gm. On March 3, 1936, the acid secretion fifteen minutes after 1 mg. histamine, was 50 clinical units free, 100 clinical units total. There was no response to alcohol. On April 18, the red count was 7.25, Hb. 13 gm. About one year postoperative, Sept. 9, 1936, twenty-four hours after ingestion of 250 gm. of dog food, no residue was found in the stomach; bile was present. Similar absence of residue occurred after this test in the remaining animals of the series. September 25, the dog passed barium sulfate in the stools four hours after its introduction by stomach tube. Beginning November 11, a solution of liver extract equivalent to 15 gm. of fresh liver was given intravenously for five days. Neither this dog nor the others in the series showed any reticulocyte changes. On Feb. 20, 1937, fifteen months postoperative, the emptying time of the standard meal was four hours, ten minutes (normal five-plus hours). On April 4, the response thirty minutes after subcutaneous histamine was 50 clinical units free and 55 clinical units total acid. The Price-Jones curve yielded a mean red cell diameter of 6.63 μ (lower normal). The slide showed no evidence of anisocytosis, poikilocytosis, or polychromatophilia. A biopsy of the femoral shaft bone marrow on Dec. 1, 1937, twenty-seven months postoperative, showed scattered areas of very slight activity. Weight at present 42 pounds.

Dog No. 123.—Operated upon Dec. 6, 1935; weight 25 pounds. Recovery was surprisingly rapid. The red cell count on December 11 was 4.58, Hb. 8.6 gm. Until one year later there was no gastric secretion to either alcohol or histamine. On December 24, 1936, the gastric sample showed 20 clinical units free and no combined acid thirty minutes after subcutaneous injection of 1 mg. histamine. On Feb. 20, 1937, the standard meal had left the stomach in three hours and fifteen minutes. On April 3, the mean red cell diameter was 6.48 μ, which is slightly smaller than the normal. There was no anisocytosis, poikilocytosis, or polychromatophilia. On November 4, the blood count showed 6.98 million red cells and 13.5 gm. hemoglobin. On Dec. 6, 1937, two years after the operation, the dog was sacrificed, weight 32.3 pounds. Autopsy showed about one-fourth to one-third of the stomach present. The choledochojejunal junction appeared anatomically reconstructed, with no dilatation of the biliary passages. The femoral bone marrow was grossly and microscopically fatty.

COMMENT

The failures of other investigators as well as ourselves to produce experimental pernicious anemia, suggests utilization of an entirely different approach in solution of the problem. Theoretically we should have succeeded if Meulengracht's hypothesis[4] is applicable to the dog. From his experiments on hog tissues he has submitted the hypothesis that the intrinsic factor of Castle originates in the so-called "pyloric gland organ," which includes the pyloric and cardiac glands of the stomach and Brunner's glands in the small intestine. Operative procedures which remove this area should produce pernicious anemia experimentally. Acting upon this suggestion, Petri and co-workers[9] removed the stomach and suprapapillary portion of the duodenum from dogs (in which Brunner's glands are said never to extend beyond the ampulla of Vater), with negative results. In our dogs we removed gastric tissue well beyond the limits of the pyloric glands, as measured by Lim,[3] who also found that the cardiac glands in dogs' stomachs are relatively sparse. We assumed, therefore, that we have removed practically the entire "pyloric gland organ."

Pernicious anemia has not appeared in gastrectomized dogs who have also had extensive enterectomy and pancreatectomy.[14] Schemensky's[12] report on the pernicious anemia properties of the hog colon would perhaps indicate that

the entire gastrointestinal tract is active. These observations support the opinion that the loss of hemopoietic elements from the human subject in pernicious anemia patients may be due to a disturbance or deficiency elsewhere in the body. Vitamin B deficiency studies reported by Miller and Rhoads,[5] and by Wills,[15] appear most significant in this connection. Unfortunately, their work could not be confirmed by Wigodsky, Bussabarger, and Ivy.[14]

SUMMARY

Seven dogs were observed for a period of over two years following radical subtotal gastrectomy, complete duodenectomy, and partial jejunectomy. None of these animals have shown any evidence of pernicious anemia. The pyloric gland organ in the dog is not essential for maintenance of normal dog blood pictures.

REFERENCES

1. Bachrach, W. H., and Fogelson, S. J.: The Effect of Antipernicious Anemia Substances Upon Guinea Pig Reticulocytosis and a Review of the Literature, J. Lab. & Clin. Med. **22**: 925, 1937.
2. Ivy, A. C., Richter, O., and Kim, M. S.: Some Experiments Bearing on the Etiology of Pernicious Anemia, Am. J. Physiol. **101**: 59, 1932.
3. Lim, R. K. S.: The Gastric Mucosa, Quart. J. Micr. Sc. **66**: 187, 1922.
4. Meulengracht, E.: Weitere Untersuchungen über Pylorusdrüsen und Brunnersche Drüsen in ihrer Beziehung zur perniziösen Anämie, Ztschr. f. klin. Med. **130**: 468, 1936.
5. Miller, D. K., and Rhoads, C. P.: The Experimental Production of Loss of Hematopoietic Elements of the Gastric Secretion and the Liver in Swine With Achlorhydria and Anemia, J. Clin. Investigation **14**: 153, 1935.
6. Mullenix, R. B., Dragstedt, C. A., and Bradley, J. D.: Hemoglobin Regeneration in Gastrectomized Dogs, Am. J. Physiol. **105**: 443, 1933.
7. Necheles, H., and Scheman, L.: Detection of Free Acid in Patients With Suspected Anacidity, J. A. M. A. **103**: 107, 1934.
8. Palmer, W. L. Quoted by Bussabarger, R. A.: A Study of the Effect of Gastrectomy in Immature Dogs, Doctor's Thesis, Northwestern University, 1937.
9. Petri, S., Bøggild, D., and Ohlsen, A. S.: Experimentelle Untersuchungen über gastrogene Anämien (bei Hunden), Hospitalstid. **79**: 509, 1936.
10. Richter, O., Ivy, A. C., and Meyer, A. L.: Study of Dog's Stomach and Liver for Substances Effective in Pernicious Anemia, Proc. Soc. Exper. Biol. & Med. **31**: 550, 1934.
11. Rosahn, P. D.: A New Capillary Hematocrit, Proc. Soc. Exper. Biol. & Med. **28**: 491, 1931.
12. Schemensky, W.: Zur Pathologie der perniziösen Anämie. Therapeutische Erfolge mit Verfütterung getrockneten Schweinedickdarmpulvers, Ztschr. f. klin. Med. **128**: 428, 1935.
13. Strauss, M. B., and Castle, W. B.: Amount of Material Effective in Pernicious Anemia Present in Dog Liver, Proc. Soc. Exper. Biol. & Med. **31**: 360, 1933.
14. Wigodsky, H. S., Bussabarger, R. A., and Ivy, A. C.: Personal communication.
15. Wills, L., and Bilmoria, H.: Studies in Pernicious Anemia of Pregnancy, Production of Macrocytic Anemia in Monkeys by Deficient Feeding, Indian J. M. Research **20**: 391, 1932.

STUDIES IN CONVULSANT THERAPY*

II. The Role of Alkalinization

Stanley Rochelle Dean, M.D., Newtown, Conn.

INTRODUCTION

THE past few years have introduced a new era in the treatment of schizophrenia, the full import of which still remains to be realized. Unlike the haphazard and multifarious methods that have been employed since antiquity to "shock patients into reality," concerted efforts are at last being made to view the problem of irritative or shock therapy in the sober light of scientific investigation.

Sakel's treatment with insulin shock and von Meduna's treatment with metrazol convulsions are the two most important methods in vogue today, and in both of these the reported results have thus far been comparable. Metrazol (pentamethylentetrazol), however, by virtue of its greater safety and ease of administration, has created widespread interest during the relatively short time that has elapsed since its introduction in this country. Because of its growing importance, it is essential to scrutinize this drug in the utmost detail, not alone from a clinical, but also from a more basic experimental point of view.

For several months our investigations have been directed toward various agents that might be synergistic, antagonistic, or neutral to the convulsant action of metrazol. The present report deals with the effects of diet and the alkaline state.

Friedman,[1] in his earlier work in this country, stressed the importance of thorough alkalinization and hydration as an adjunct to convulsant therapy, basing that procedure upon the fact that the alkaline state is tetanic or convulsive in itself and should, therefore, assist in the maintenance of an irritative background in the central nervous system; thus, theoretically, the convulsive threshold would be lowered, less metrazol would be required to produce a paroxysm, and the number of grand mal attacks would be materially increased. He set the minimal fluid intake at two liters per day, and suggested the use of a high alkaline-ash diet supplemented by sufficient alkali drugs to maintain the urinary reaction constantly blue to litmus paper.

These principles are, of course, the converse of those utilized in the treatment of epilepsy, i.e., with ketogenic diets and dehydration, and, theoretically at least, appear to be sound. From a practical viewpoint, however, we found the procedure to be undesirable for the following reasons:

1. High alkaline-ash diets are objectionable to patients and impose an added burden upon the hospital.

*From the Fairfield State Hospital, Newtown.
Received for publication, April 25, 1938.

2. The simultaneous exhibition of two potent alterative agents (in this case alkalosis and metrazol) makes it more difficult to evaluate results from a scientific point of view than if one agent alone were used.

3. Prolonged alkalinization may lead to alkalosis, with varying degrees of toxicity in susceptible individuals. Among the indicia described by Ellis,[2] Koehler,[3] Gatewood and his co-workers,[4] and numerous others, as resulting from excessive alkali ingestion, are headache, drowsiness, anorexia, nausea, vomiting, myalgias, nervousness, mental depression, fatigue, faintness, irritability, vertigo, delirium, and tetany.

It seemed to us, therefore, that it would be of decided value to determine just how necessary, if at all, was the role of alkalinization in convulsant therapy.

PROCEDURE

The description of metrazol, its mode of administration, and clinical effects have been the subject of another paper.[5] Suffice it to state here that the routine course of treatment is conducted as follows: We begin with the rapid intravenous injection of 3 c.c. of a 10 per cent solution of the drug, and if no convulsion occurs, increase the succeeding doses by increments of 1 c.c. every other day until a grand mal seizure results. The convulsant dose having been determined (usually from 3 to 5 c.c.), it is repeated at the next treatment, and then automatically increased by 0.5 c.c. at every third injection, as long as convulsions occur in an unbroken series; this dosage plan may be indicated numerically as follows, beginning with the initial convulsant dose: 4-4-4½-5-5-5½-6-6-6½-7-7-7½-8-8, etc.

If, however, a petit mal reaction occurs at any point, the next dose should be increased by one cubic centimeter, bearing in mind the fact that the maximum dose of 15 c.c. must not be exceeded. Of course, it is not likely that this plan can be invariably followed without deviation in every case; but by adhering to it as rigidly as possible, it is often possible to keep a step in advance of the patient's tolerance, so to speak, and thereby reduce petit mal reactions to a minimum. Then, too, it provides one with a crude yardstick for comparing various situations; for although there is considerable individual variation in the response to metrazol, the use of a standard procedure in hundreds of injections results in a fairly constant ratio of grand mal to petit mal reactions.

In the present instance our study comprised a total of 636 metrazol injections, of which the first 287 were given in conjunction with the alkaline regime, whereas the remaining 349 were administered to patients who received an ordinary house diet without alkalies. For the sake of convenience we may designate the first series, Group A; the second series, Group B.

The high alkaline-ash diet used in Group A was prepared from the following prescription:*

Protein	71.2 gm.
Fat	100.9 gm.
Carbohydrate	303.8 gm.
Total calories	2,403.0
Excess acid	14.6
Excess base	100.5

*Calculated for us by Adelia Beeuwkes of the University Hospital, Ann Arbor, Mich.

The daily caloric intake, however, was not limited to the above basic requirements, the patient being encouraged to eat as much as he desired. The diet was supplemented by a palatable, effervescent citrate carbonate preparation* in amounts sufficient to keep the urine constantly alkaline to litmus paper. This alkaline regime was instituted a week before, and then continued throughout the entire course of metrazol therapy, a period of two to three months in duration.

Table I provides a comparison of some of the measurable observations in the two series.

TABLE I

	GROUP A (ALKALI) 287 INJECTIONS	GROUP B (NO ALKALI) 349 INJECTIONS
1. Ratio of grand mal to petit mal reactions	2.8:1.0	2.5:1.0
2. Average initial convulsant dose of metrazol	4 c.c.	4 c.c.
3. Average dose required to produce the twentieth convulsion	9.7 c.c.	9.8 c.c.
4. Average duration of metrazol episode (injection to resting stage)	62 sec.	65 sec.
5. Average weight gain after sixty days of treatment	2.66 kg.	3.42 kg.

COMMENT AND CONCLUSIONS

Although the observations recorded in Table I are almost self-explanatory, it might be interesting to consider them in some detail. The ratio of grand mal to petit mal reactions, which had been 2.8:1 throughout the first series of injections, dropped slightly to 2.5:1 a week after alkalinization and hydration were discontinued, and remained constant at that level throughout the second series. This drop apparently indicates a slightly diminished reactivity of Group B to metrazol, but obviously not enough to be of any practical importance.

Another observation of interest pertains to the initial convulsant dose of metrazol in each series. If alkalinization and hydration really lower the convulsive threshold, one should expect this dose to be correspondingly reduced in Group A; yet the averages of several initial convulsions indicate that the minimal dose is exactly the same in either case.

Significant as these two clues may be, a more important consideration, in our opinion, is the average dose required to produce the twentieth convulsion, this number being arbitrarily chosen as representing a late period in the metrazol regime. It is known that a patient's tolerance to metrazol gradually increases with successive injections, and that the effective amount of metrazol must, therefore, be correspondingly increased. Therefore, a comparison of average doses at the late period selected ought to demonstrate convincingly whether or not alkalinization and the so-called "irritative background" are effective in reducing tolerance. Reference to Table I definitely indicates that they are not—since the dose in each series is practically the same.

Does the alkaline state augment the duration and severity of the paroxysm? The average duration of the metrazol episode is sixty-two seconds in Group A; sixty-five seconds in Group B; the very slight difference probably falls within the limits of error. Our clinical impression is that there is actually little or no

*Citrocarbonate (Upjohn).

difference in duration or intensity. True, there is evidence of increased neuromuscular irritability in Group A, for tapping certain areas sometimes produces marked muscular twitching and jactitation; but this phenomenon seems to have no effect upon the metrazol convulsion itself.

It is interesting to note the greater weight gain in Group B. This is not hard to explain when one realizes the average patient's aversion to the alkaline diet, and the fact that anorexia is itself symptomatic of prolonged alkalinization. It is necessary to point out that this gain in weight is not limited to a sixty-day period (arbitrarily selected for convenient comparison), but may go on, in favorable cases, for several weeks or months after the course of metrazol has been completed.

Thus far we have been dealing with measurable phenomena. Equally important to us, though harder to demonstrate to others, is our clinical impression. All of us who have followed this investigation have been impressed with the advantages both to the patient and the hospital of the alkaline-free regime. Considering all these factors together, we must conclude that not only are alkalinization and hydration unnecessary, but definitely undesirable in convulsant therapy.

REFERENCES

1. Friedman, E.: The Irritative Treatment of Schizophrenia, Am. J. Psychiat. **94**: 355, 1937. Irritative Therapy of Schizophrenia, New York State J. Med. **37**: 1, 1937.
2. Ellis, A. W. M.: Quoted in the Cyclopedia of Medicine, Philadelphia, 1934, F. A. Davis Co. **1**: 313.
3. Koehler, A. E.: Ibid.
4. Gatewood, W. E., Gaebler, O. H., Muntwyler, E., and Meyers, V. C.: Ibid.
5. Dean, S. R.: Studies in Convulsant Therapy. I. Technique and Clinical Phenomena, J. Connecticut M. Soc. **2**: 325, 1938.

ABNORMAL UTERINE BLEEDING AS A SYMPTOM IN TYPHOID FEVER*

CHARLES P. WOFFORD, M.D., CLEVELAND, OHIO, AND DUNCAN G. CALDER, M.D., AND FERDINAND FETTER, M.D., PHILADELPHIA, PA.

THREE patients were admitted to the Philadelphia General Hospital within a relatively short period of time, each suffering with typhoid fever and showing in addition such abnormalities of the menstrual cycle that two of the three were first assigned to the gynecologic wards. In fact, one of the patients was thought to have had a septic abortion for several days until laboratory studies established the true diagnosis. This coincidence led us to investigate the frequency of abnormal uterine bleeding in association with typhoid fever.

There is brief mention of this symptom in the literature. McCrae[1] observes that menstruation usually "ceases during the course of the disease, although at the onset it is not uncommon for it to occur, often with a short interval since the preceding period." However, he found uterine bleeding present in only 11 of 438 women with typhoid fever, and then not in any profuse amounts. Occasional excessive uterine bleeding was described in the hemorrhagic type of typhoid fever, fortunately a very rare form of the disease. Edwards[2] states that "menstruation often appears early at the onset of typhoid" and that there is amenorrhea in some 60 per cent of cases. It is quite generally agreed (McCrae,[1] Edwards,[2] and Hare[3]) that abortion or premature labor is very frequent when pregnancy is complicated by typhoid fever.

It is, of course, well known that various menstrual disorders are associated with many infectious diseases of both the acute and chronic types. However, in view of the paucity of attention called to this symptom in typhoid fever, the following case histories are briefly summarized:

CASE 1.—E. C., white female, aged 23 years. The patient was admitted September 1, 1937, to the gynecologic wards with a history of nausea, vomiting, headache, malaise, generalized aches and pains, together with localized epigastric and lower abdominal pains, dating back eighteen days. Constipation was present until the day before admission, when it was replaced by diarrhea. Uterine bleeding and a recently irregular menstrual cycle accounted for her admission to the gynecologic department. Her menses had begun at the age of 12 years, were always regular, every twenty-eight to thirty days, lasting three days, with a moderate flow and occasional cramps on the first day. Six weeks prior to admission, July 15, her expected period began normally, but lasted six days instead of the usual three. After two days with no bleeding, the bleeding recurred and lasted another week. On August 15, another period began and lasted only two days. On August 28, four days

*From the Medical Service of Dr. Truman G. Schnabel, Philadelphia General Hospital, Philadelphia.

Read at the Section on General Medicine of the College of Physicians of Philadelphia, Pa., April 25, 1938.

Received for publication, May 7, 1938.

before admission, metrorrhagia again appeared, associated with the passage of numerous clots, some fairly large in size. A steady, small flow continued until her arrival at the hospital. Systemic review, previous medical history, family history, and social history, added nothing of significance, save for one normal pregnancy several years ago.

On physical examination the young woman did not appear acutely ill in spite of a temperature of 104° F. The pulse was 124. No rose spots were visible. The tongue was heavily coated and the pharynx injected. Examination of the cardiovascular and respiratory systems revealed no abnormalities. Pelvic examination showed slight uterine bleeding, a cyanotic cervix and vaginal vault, and an external os which admitted one finger. The adnexae were normal. The uterus was retroflexed, movable, but not increased in size nor softened in consistency. No other pertinent physical findings were noted. Uterine bleeding stopped within a few hours after admission, only to recur four days later and last two days, with the passage of numerous clots.

The true diagnosis was reached only after a week, during which time she had been treated with blood transfusions and other appropriate measures for a possible septic abortion. On the seventh day after admission—the twenty-fifth day of her disease—she developed rose spots, and the blood culture was reported positive for *B. typhosus,* 12 colonies per 3 c.c. of blood. The Widal reaction showed an agglutination in a dilution of 1:640 for the H antigen, and 1:320 for the O antigen. It was negative for *paratyphosus A* and *B* and *Brucella abortus.* The leucocyte count was consistent with typhoid fever, 7,200 per c. mm. on one occasion, and 8,700 per c. mm. on another, with a differential in the former case of 82 per cent neutrophiles, 12 per cent lymphocytes, 4 per cent monocytes, and 2 per cent basophiles, and a Schilling index of 40 and a multiple index of 640.

She was transferred to the medical service of Dr. Truman G. Schnabel on September 8, and her subsequent course was uneventful. Her temperature became normal by September 10 and remained so except for a brief elevation five days later when it reached 100° F. During her hospitalization there was nothing further to indicate the possibility of an abortion, and it was obvious that this initial diagnosis was erroneous. She was discharged from the hospital on October 5, 1937. Just before her discharge, October 3, another menstrual period started. This lasted until October 17 and was associated with severe cramps, but no clots. The bleeding each day was more profuse than normal. Again on November 10, a menstrual period began and lasted seven days, with moderate cramps. Otherwise she felt well since leaving the hospital.

Case 2.—A. C., negro female, 18 years of age. The patient was admitted August 23, 1937, to the medical service of Dr. Truman G. Schnabel, with symptoms of only three days' duration, namely, headache, nausea, repeated vomiting, anorexia, and lower abdominal pain. A nonproductive cough was present. In the systemic review no further points were of interest save her menstrual history. Her menses began when she was 14 years old. They were always regular, every thirty days, lasting two to four days. Her last period began July 25, lasted three days and was normal in all respects. She had had one full-term pregnancy, ending in delivery in February, 1936.

Physical examination on admission showed an acutely ill colored girl, with a temperature of 104° F. and a pulse of 100. The breath was foul, the tongue was coated, and the tonsils and pharynx injected. Examination of the chest revealed some impairment to percussion in the left lower lobe, with slight suppression of breath sounds. The cardiovascular system was normal. Abdominal palpation elicited generalized tenderness, and there were moderate distention and increased, high-pitched peristalsis. Pelvic examination showed a small amount of uterine bleeding, of which the patient was unaware, and a cervix which was lacerated from her previous pregnancy. The uterus was small and slightly anteverted. Pain was elicited on manipulation of the cervix.

The clinical diagnosis of typhoid fever was confirmed by the laboratory three days after admission, with the report of a positive urine culture for *B. typhosus,* and further substantiated two days later by a positive blood culture for the same organism, 2 colonies

per 3 c.c. of blood. The Widal reaction, however, was never positive in a higher dilution than 1:160 in the O antigen, and was negative in the H antigen during her entire stay in the hospital.

Three days after admission, the sixth day of her disease, she began passing numerous clots of blood from the vagina. This was thirty-two days after her last period. On August 28, more than 8 ounces of clotted blood were evacuated at one time. The clots were interspersed with a foul-smelling, reddish-brown vaginal discharge. All bleeding had stopped by September 2, ten days after admission. There was no subsequent bleeding while on the wards. Her clinical course in the hospital was stormy, complicated by an acute suppurative parotitis, which required surgical intervention. She recovered satisfactorily and was discharged in good condition on October 29, 1937.

A follow-up examination on December 7, 1937, found the patient in good physical condition. She had gained over 15 pounds in weight and was symptom free. She stated, however, that she had had no uterine bleeding since September 2, save for slight spotting for one hour on November 28. Pelvic examination failed to reveal any evidences of pregnancy or gross abnormalities.

CASE 3.—M. F., white female, aged 35 years. This patient's illness was ushered in by severe frontal headache and uterine bleeding seven days before admission to the gynecologic wards of the hospital on October 11, 1937. She had been well until that time, but on awakening on the morning of October 4, she had such a severe headache that she fainted on the way to the bathroom. Profuse uterine bleeding made its appearance almost immediately and continued for two days. There was no bleeding thereafter, even during her entire hospital course. Diarrhea, anorexia, and occasional vomiting were also experienced during the week prior to admission. Her menses had begun at the age of 11 years, were always regular every thirty days, lasting four to five days. After missing three periods, she had a self-induced abortion in May, 1937, and had a totally irregular menstrual cycle after that episode, characterized by occasional spotting, occurring every two to three days, filling one pad, but with no normal flow. The patient had four living children and two abortions, the last as mentioned above. No other points in the history were significant.

On admission the patient appeared acutely ill, with a temperature of 104.6° F. and a pulse of 88. She had an intense headache, was sullen but cooperative. The tongue was heavily coated, the breath fetid, and the pharynx injected. The abdomen was slightly distended, and peristalsis was hyperactive. Rose spots were scattered over the abdomen. Pelvic examination was negative. There was no uterine bleeding at that time. The remainder of the physical examination likewise revealed no pertinent findings.

The blood count on admission showed: R.B.C. 4,780,000 per c. mm., Hb. 71 per cent, W.B.C. 6,700 per c. mm., neutrophiles 64 per cent, lymphocytes 28 per cent, monocytes 8 per cent, with a Schilling index of 4.3 and a multiple index of 68. Subsequent blood counts showed the same general picture, but with an increasing left shift. A blood culture on October 12 was positive for *B. typhosus*, 4 colonies per 3 c.c. of blood, and the Widal reaction was positive in a dilution of 1:320 for the O antigen and 1:160 for the H antigen. In spite of treatment, which consisted of blood transfusions, parenteral fluids, and other appropriate measures, her subsequent course was a downhill one, complicated by bronchopneumonia and pyelonephritis, and ended with her death on November 2, 1937.

Post-mortem examination revealed myocardial degeneration, bronchopneumonia in the right lower lobe with passive congestion and edema, and a spleen which was the seat of diffuse hyperplasia. There was hyperplasia of the mesenteric lymph nodes. Numerous typhoid ulcers were found in the ileum, cecum, and colon. The liver showed fatty infiltration and parenchymatous degeneration. The latter change was also present in the pancreas. There was lipoid depletion of the adrenals, as well as medullary degeneration. The kidneys showed bilateral pyelonephritis. There was also an ureteritis and hemorrhagic cystitis. The uterus was small, firm, and in normal position. The endometrium was slightly thickened and bloody. No evidence of placental tissue was found on either gross or microscopic examination.

COMMENT AND SUMMARY

Considering the fact that each of these three patients showed irregularities of the menstrual cycle during her illness, it seems appropriate to stress this symptom as one which may be found at the inception or during the course of typhoid fever. The irregularity was different in each case. In one it took the form of prolonged, excessive metrorrhagia, with the passage of numerous clots during the first and second weeks of her disease. In the second patient the symptoms were those of menorrhagia, together with a change in the character of the menstrual flow, which became foul-smelling, reddish-brown, and occasionally interspersed with clots. In the last case the menstrual cycle had been disrupted by an abortion five months previously, but her typhoid fever was ushered in by unusually profuse uterine bleeding.

We believe that the excessive uterine bleeding in these patients was simply part of the disease, and that it does not represent the extremely rare hemorrhagic type of typhoid fever mentioned by Osler and McCrae.[1] Unfortunately, no bleeding or clotting times or platelet counts were done on these patients. The absence of other hemorrhagic phenomena and the subsidence of the bleeding, however, indicate that a blood dyscrasia was not present. The importance of the symptom lies in the possibility of its confusing the diagnosis, as shown strikingly in the first case cited. This error might be avoided in similar instances if the possibility of menorrhagia and metrorrhagia in typhoid fever is kept in mind. It is our opinion that abnormal uterine bleeding in typhoid fever is encountered more frequently than the brief mention in the literature would indicate.

REFERENCES

1. McCrae, Thomas: Osler's Modern Medicine, ed. 3, Philadelphia, 1925, Lea & Febiger, pp. 133, 144.
2. Edwards, A. R.: Principles and Practice of Medicine, ed. 3, Philadelphia, 1916, Lea & Febiger, p. 40.
3. Hare, H. A.: Practice of Medicine, ed. 2, Philadelphia, 1907, Lea Brothers & Co., p. 45.

DISSECTING ANEURYSMS OF AORTA*

HERBERT J. SCHATTENBERG, M.D., AND JOSEPH ZISKIND, M.D., NEW ORLEANS, LA.

A DISSECTING aneurysm usually begins as a tear through the inner layers of a vessel wall, dissects between its layers, and forms a longitudinal and circular cavity. It is, however, believed by some writers, namely, Tyson,[1] Babes and Mironescu,[2] Whitman and Stein,[3] and Krukenberg,[4] that a tear in the intima is not a necessary part in the formation of dissecting aneurysms. They hold that it begins with a rupture of the vasa vasorum into a weakened media with the formation of a hematoma that splits the fibers of the media. These observers, therefore, believe that when a tear in the intima occurs, it is most likely secondary to the formation of the dissecting aneurysm.

In almost all cases the vessel involved is the aorta, but dissecting aneurysms can form in smaller arteries. This aneurysm may perforate to the exterior by tearing through its outer wall, or it may less frequently rupture back into the lumen of the vessel. In the latter case recovery may take place because of the formation of a canal lined by endothelium, through which the blood will circulate. This latter result does not invariably prevent rupture to the exterior. It is, however, very important in the healing process of dissecting aneurysms. It is aided by the fact that the canal passes through tissues that are well supplied with blood vessels, especially on the outer aspect from which organization can take place. Very rarely the blood within the aneurysm will organize, and the channel will be completely obliterated.

Dissecting aneurysm is a relatively rare condition. Walker and Walker[5] found an incidence of one rupture in 2,500 necropsies, and Ames and Townsend[6] found this condition once in 500 autopsies. Weiss[7] has found a ratio of 1:320 in adults.

This disease is usually found in individuals above the age of 40, and the relative incidence increases with age. However, instances of rupture may occur at any age. Klotz and Simpson[8] collected 42 cases of spontaneous rupture of the aorta in individuals under 40. Of these, 2 cases occurred between the ages of 1 and 10, 7 were found between 11 and 20, 20 between 21 and 30, and 13 between 31 and 40. It is also quite interesting to note that it occurs twice as often in males as in females.

The intimal tear may be longitudinal, oblique, or angular, and in some cases has been found to be multiple. It is often transverse and irregular, and measures from 1.5 cm. to 2 cm. in length. This tear in many cases lies about 1.5 cm. above the aortic valve ring. In about 70 per cent of cases the intimal

*From the Departments of Pathology, Tulane University School of Medicine and the State Charity Hospital of Louisiana.

Received for publication, May 9, 1938.

tear is in the ascending aorta. The remaining 30 per cent is found in the transverse and descending aorta, with more cases in the transverse portion. The dissecting plane is almost always between the middle and outer thirds of the media and involves approximately one-half to two-thirds of the entire circumference. The length of the sac varies from a few centimeters to almost the entire length of the aorta.

In most cases death is due to secondary rupture of the aorta. Approximately 70 per cent of cases rupture into the pericardial sac, and the remainder rupture into the left pleural cavity, the mediastium, the right pleural cavity, the abdominal cavity, or the retroperitoneal tissue in that approximate order of frequency.

Since the first description of a dissecting aneurysm by Nicholls[9] in 1762, the pathogenesis of this condition has been in dispute. Various theories have arisen which may be classified under four headings, namely, mechanical, inflammatory, degenerative, and congenital.

Mechanical.—Some authors, such as Boström,[10] Busse,[11] Oppenheim,[12] and Samson,[13] believe that a definite external trauma can be frequently elicited in history.

Boström[10] maintains that a mechanical trauma is the only factor in the formation of both the primary and secondary ruptures. Rindfleisch[14] claims that certain bands pass directly from the main pulmonary artery to the aorta and by holding these together about 1 cm. from the origin of the vessels, aided by the ligamentum arteriosum, one can determine the usual sites of the primary tears.

It has been generally agreed upon by many observers that forces which cause a sudden cardiovascular strain and increase in blood pressure will not injure the normal aorta. Many believe, however, that such forces may cause the formation of a dissecting aneurysm in persons having congenital cardiovascular anomalies.[15, 16] Oppenheim[12] observed that rupture of the aorta will not occur until a pressure of approximately 3,000 mm. of mercury is reached. This is about twenty times that of the normal pressure.

Samson[13] states that traumatic aneurysms are practically all dissecting. He believes that this type is on the increase because of the more frequent number of industrial and automobile accidents and that they are formed at the time of the accident or immediately following it. The intimal tear is located at or near the attachment of the obliterated ductus botalli, or just above the aortic valves on the posterior part of the wall of the aorta. Samson[13] argues further that traumatic dissecting aneurysms may occur in a healthy aorta, providing the causative force is severe enough and, that, under certain conditions the healthy aorta may be torn completely across.

Weiss[7] claims that the majority of traumatic ruptures of the aorta are not the dissecting type, but that the sudden and severe external force exerted on the noncompressible column of blood will cause a complete tear of the vessel.

Shennan[17] is in agreement with this latter view, that an external force causes complete rupture of the aorta. He does not agree that mechanical injuries, whether external or internal, are very important factors. He does believe, however, that a sudden increase of blood pressure, usually caused by a physical or mental strain, is important only when there is disease of the vessel wall. Far more important to this observer[18] than the increase of the systolic pressure is the abrupt diastolic recoil. In most of Shennan's cases hypertension associated with an hypertrophied left ventricle was present, but in about one-fifth of the reported cases no hypertension was noted.

Inflammatory.—Rokitansky[19] appears to be the first to have considered the inflammatory theory. He stated that in one form of dissecting aneurysm the adventitia is easily separated from the media as a thick, vascular layer in a state of chronic inflammation. However, later authors who think that inflammation is a causal factor in dissecting aneurysms believe that it is the media that is involved. To Köster[20] is usually given the credit for the inflammatory theory. His view is that a mesarteritis extends from the adventitia along the vasa vasorum and that this causes fibrosis and destruction of the muscularis and elastica. This weakens the media and allows for rupture when the blood pressure is raised.

Babes and Mironescu[2] have described degenerative changes with infiltrating cells and with new vessels which, by rupture, will form small hemorrhages in the media. These, therefore, do not arise from the lumen of the artery. To this process they have given the name of "dissecting aortitis." The findings have been corroborated by Krukenberg,[4] Tyson,[1] Whitman and Stein,[3] and others.[21-23] These observers, therefore, concluded that a dissecting aneurysm may form in the absence of a tear of the intima.

Certain specific diseases, such as syphilis and rheumatic fever, have occasionally been considered as being important in the causation of dissecting aneurysm. Syphilis, however, does not appear to be a factor in their production. Klotz and Simpson[8] state that the very nature of the syphilitic process with its gumma and fibrosis does not allow for dissection. In fact, they believe that the granulomatous inflammatory invasion of the arterial wall will tend to hold the neighboring laminae more closely together, so that the wall may be less readily split into its anatomic layers than normally.

Shennan[17] claims that there is some resemblance of the medial and adventitial reactive changes to those described in rheumatic aortitis. It, therefore, seems possible to him that in this disease, degenerative atrophic and acute necrotic changes may be produced in the media which are similar to those caused by other toxins.

Degenerative.—According to Virchow and a number of other pathologists,[24, 25] dissecting aneurysm was thought to be formed by the dissection of blood through the base of an atheromatous ulcer or its border into the layers between the media and intima or between the media and adventitia. This is probably true for a small number of cases, but it is in the ascending aorta, where the aorta is relatively immune to arteriosclerosis, that most primary ruptures are found.

Peacock[26] believed that degenerative changes in the aortic wall were important, especially when they involved the media. The importance of microscopic interruptions or "faults" in the media was emphasized by Shennan and Pirie.[18] These were associated with atrophic changes, partial or complete disappearance of muscle fibers in large areas of the media, and degenerative changes in the connective tissue and elastica. These findings were also noted by Moriani.[27]

Gsell[28] found that the media had undergone noninflammatory necroses in his cases. He thought that these lesions were similar to those described by Wiesel[29] who had observed peculiar focal necroses in the vessel walls of young individuals dying from acute infections.

Erdheim,[30] in his study of dissecting aneurysms, found lesions in the aorta which were similar to those described by Gsell. He observed small areas of necrosis and peculiar hyalinized vacuoles in the media beyond the region of the rupture. The elastic, muscular, and connective tissue elements were involved. No inflammatory reaction and no reparative process were noted. These necroses were most numerous in the outer two-thirds of the media. This entire destructive process in the media caused necrosis of the cellular elements, and this disintegrated material was absorbed without local tissue reaction. These areas were then filled with an albuminous fluid, which then formed the picture of peculiar cystic spaces between elastic fibers. This author felt that the process of necrosis and that of mucoid degeneration were different. This peculiar medial degeneration was also found more frequently in older individuals, aside from those cases having dissecting aneurysms. It was either diffuse or patchy. As to the etiology of these changes, Erdheim is unable to say, but he believed that the action of toxins is highly important. Klotz and Simpson[8] and Moritz[31] in later studies have found changes in the aorta similar to those described by Erdheim.

Congenital.—Many cases of dissecting aneurysm in young persons have some congenital changes in the aorta.[15, 16] The most important are coarctation and dilatation of the aorta, with thinning of the wall. Other congenital factors are a thymicolymphatic constitution, with aortic and cardiac hypoplasia and aortic stenosis.

The two cases here reported show changes in the media of the aorta similar to those described by Erdheim.

CASE 1.—A. D., a colored male, aged 23 years, was admitted to the hospital on April 23, 1933, with complaints of cough, pain in the right side, difficulty in breathing, and swelling of the legs. The patient stated that he had had gonorrhea, and "pimples" on the meatus of the urethra of three weeks' duration about a year and a half previously. He denied having a chancre. He had pneumonia in January, 1933, and was confined to bed for nineteen days, after which the least exertion produced dyspnea. The following month he was forced to go to bed again because of dyspnea, cough, and edema of the lower extremities, and severe pain in the right side of the abdomen. Subsequent to the attack of pneumonia and the sequelae, the patient never regained his former health, spending most of his time in bed.

Physical examination revealed a fairly well developed and nourished colored male, with edema of the entire body, who appeared critically ill. Ascites also appeared to be present. The chest was of the asthenic type and breathing was labored. The lungs showed impairment

of resonance at right base posteriorly. In both bases posteriorly, crepitant and subcrepitant râles were heard. The heart was markedly enlarged. The right border was one inch to the right of the sternal margin in fourth right interspace and the left margin extended out to the left axillary line. There was an intense thrill during diastole which was felt over the entire precordium. A loud high pitched diastolic murmur was heard over the entire precordial area. The pulmonic second sound was extremely loud and had a metallic quality. The blood pressure was 140/40. The Wassermann reaction was negative. The urinalysis showed a moderate amount of albumin and a few hyaline and granular casts. The patient died shortly after admission.

Autopsy: The body was that of a well-developed well-nourished negro male. The skin was smooth and tense, due to the edematous condition of entire body. The pupils measured 4 mm. and were equal. No penile scars were noted. The pleural cavities contained approximately one liter of blood-tinged fluid. The left lung was adherent to the diaphragm below.

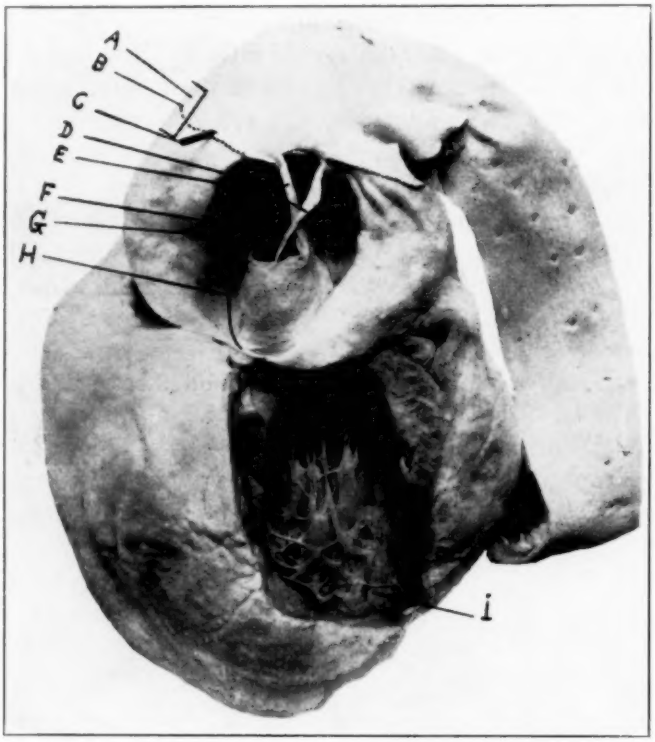

Fig. 1.—Photograph showing dissecting aortic aneurysm presenting the following details: *A*, Area selected for histologic section, upper portion of which contains all three layers of aortic wall and lower portion which contains only adventitia and part of media (see Fig. 2). *B*, Dotted line represents course of tear which involved almost entire circumference of vessel. *C*, Probe indicating minute intimal depression and tear which permitted blood to extravasate from aorta into aortic wall, with subsequent splitting of media into two separate portions. *D*, Aneurysmal dilatation, wall of which consists of adventitia, outer portion of media and newly formed connective tissue replacing intima and greater part of media. *E*, Strips consisting of intima and inner portion of media forming typical "X." These structures connect the upper normal portion of aorta with the lower retracted portion "H." *F*, Probe inserted in small 5 mm. tear in adventitia and outer part of media. It is at this point that hemmorrhage took place into the pericardial cavity. *G*, Tubing inserted to show normal passage from left ventricle through aortic valve and conical structure "H" into aneurysmal dilatation "D." *H*, Conical projection, 2 cm. in height and 1.5 cm. in diameter, consisting of intima and inner portion of media attached to aortic ring below, only remaining part of proximal portion of aorta not dilated. *I*, Left ventricular cavity.

The right lung showed numerous adhesions to the ribs anteriorly. On section both lungs were markedly congested. The liver weighed 1,860 gm. The cut surface had a typical "nutmeg" appearance. The spleen weighed 250 gm. and revealed marked congestion. The intestines, stomach, pancreas, adrenals, and kidneys showed considerable congestion.

The pericardial sac was greatly distended and filled a good portion of the left chest cavity. On opening the sac, 970 c.c. of clotted blood was found.

The heart had a marked increase in the amount of subepicardial fat and weighed 250 gm. The pulmonic valve measured 6 cm., the mitral valve 10 cm., and the tricuspid valve 12 cm. The aortic valve was not opened in order that the relationship of the pericardial hemorrhage to this area might be studied. The left ventricular wall measured 1.5 cm., and the right measured 0.5 cm.

Aorta: Externally for a distance of 5 cm. the proximal portion of the ascending aorta appeared dilated. Upon opening this dilated structure it was found that the vessel wall in this area had been broken by a dissecting aneurysm. Apparently the primary break had its beginning as indicated by *C* shown in Fig. 1. This opening led into the media, but did not penetrate through the aortic wall. It was through this small aperture that blood from the aorta proper infiltrated between the medial layers dissecting toward the adventitia and left the greater part of the elastic sructure to the luminal side of the vessel. Blood, evidently, traversed the wall, and the entire conical protrusion at *H* was torn loose, with the exception of the two strips in an X-line arrangement, which continued intact. An almost complete circular tear, therefore, involving the intima and a portion of the media occurred at this primary injury. This break apparently weakened the vessel at this point, with a resultant bulging or aneurysmal formation of the remaining unbroken portion of media and

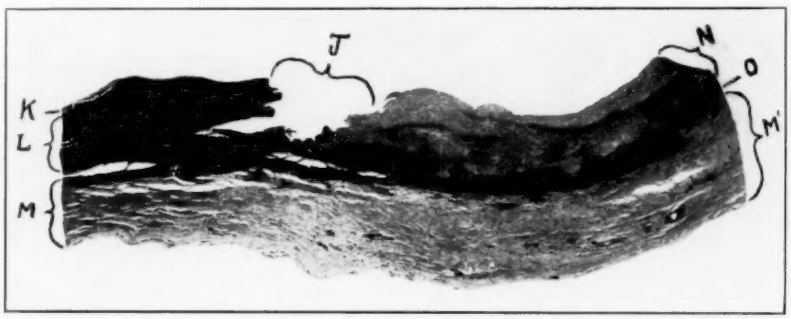

Fig. 2.—Section of area *A* shown in Fig. 1 (low power): *J*, Point at which primary tear and dissection took place. The inner media and intima were torn loose at this place to form the conelike protrusion H described in Fig. 1. *K*, Shows normal intact intima. *L*, Showing appearance of media a short distance distal to tear. *M*, Adventitia. *M'*, Adventitia. *N*, Shows marked proliferation of newly formed edematous connective tissue lying upon remaining portion of media. This newly formed connective tissue constituted the inner lining of the dissecting aneurysm. *O*, Outer portion of media forming a dark line, extending through section and continuous with outer portion of "L."

adventitia. This bulging did not include the aortic ring which remained intact within certain portions of the inner aortic coats (intima and part of media). From the aortic ring these coats protruded upward into the dilated aorta for a distance of 2 cm. in a conical form. From the ends of this conelike protrusion, the two stripped portions of the aortic wall connected in an X form with the unbroken aorta above, as shown by *E* (Fig. 1). A careful examination for a possible rupture of the remaining outer portion of vessel wall was made, and a short linear tear, 0.5 cm. in length, was found as indicated by *F*. It was at this point that hemorrhage took place into the pericardial cavity, causing the sudden death of the patient.

A further examination of the aorta, extending from the distal portion of the aneurysmal formation down to and including the bifurcation of iliacs, showed only an occasional yellowish elevated area. These lesions were located especially in the thoracic aorta near the openings of the intercostal arteries.

Case 2.—This specimen of a dissecting aneurysm was received by us through the courtesy of Dr. J. E. Robinson of Temple, Texas. A. F., a white male, 56 years of age, was admitted to the hospital with a history of a tearing pain in the chest. This occurred immediately following a meal, and he gradually went into shock. He was treated with morphine for his

pain but died suddenly, twelve hours after his admission. His blood Wassermann was negative, and his past history revealed that he suffered from "rheumatism" and "stomach trouble" for a number of years.

Autopsy: The body was that of a well-developed and well-nourished white male, weighing 210 pounds and measuring 61 inches in length. The heart was surrounded by a large

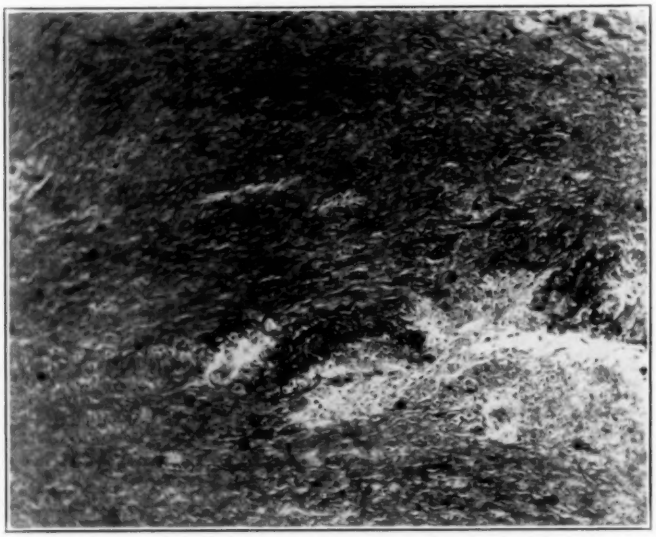

Fig. 3.—An area of medial degeneration with some evidence of healing by fibroblasts and cyst formation.

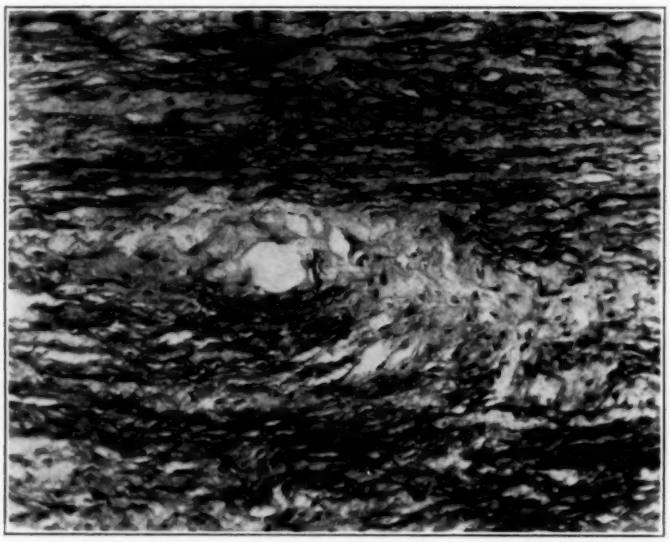

Fig. 4.—High power view showing cystic area and spaces between elastic fibers containing a mucinlike substance. Considerable destruction of elastic lamellae is also present.

amount of freshly clotted blood. The heart was enlarged and weighed 460 gm. A moderate amount of fat was present in the subepicardium. The valves were essentially normal. The left ventricular wall measured 2.2 cm. in width. The myocardium was firm, reddish brown in color, and showed no areas of fibrosis. The coronary arteries were patent and showed an occasional yellow atheromatous plaque.

Aorta: A transverse tear, 3 cm. long, was noted through the intima and media about 2 cm. above the aortic valves. The edges of the tear were sharp and a dissecting aneurysm began here and undermined its way for about 7 cm. above the tear and downward into the pericardial cavity through a laceration, 0.7 cm. in length.

Microscopic Findings: The histopathologic findings were similar in both cases and will be described together. In Case 1, the lesions were most pronounced in the ascending aorta while in Case 2, they were seen throughout the vessel.

The intima in Case 1 in the region of the tear was normal. The aneurysmal wall showed newly formed connective tissue (Fig. 2), which replaced the original intima and inner media. The intima in Case 2 near the tear showed a slight increase in the subendothelial connective tissue but otherwise the changes were minimal.

The most characteristic lesion consisted of patchy or diffuse necroses in the media. These were found distributed in a general manner, but were most commonly seen in the region of the junction of the middle and outer thirds of the media. No exudative reaction was seen in the neighborhood of these necrotic areas, but some defects were seen undergoing repair by a slight fibroblastic proliferation (Fig. 3).

The muscle fibers and elastic laminae were involved in this process, but the destruction of the former structures was more marked. The muscle fibers showed altered nuclei, fragments of nuclei, or had undergone complete dissolution. Clefts or cysts filled with a mucinlike material were, therefore, seen between elastic fibers (Fig. 4). Generally this mucinlike substance could occasionally be seen between the muscle and elastic fibers without cystic formation. With the increase of this mucinlike material there was a decrease in the number of interlamellar muscle and connective tissue cells. At times the elastic fibers were damaged, as evidenced by fragmentation, irregular thickenings, and loss of normal staining reaction. Occasionally these were completely destroyed. No unusual fatty changes could be seen in the media.

Very little compensatory adventitial thickening was noted in either case. The vasa vasorum in Case 2 showed occasional moderate intimal proliferation, without perivascular infiltration.

DISCUSSION

In both cases there were lesions in the media of aorta, especially in the region of the rupture, which were thought to be responsible for the tear in the weakened wall.

The microscopic changes in the media noted in these cases are similar to those described by Gsell and Erdheim. This consists of a peculiar degeneration of the media not associated with a significant intimal or inflammatory reaction. With the degeneration of muscle, connective tissue, and occasional elastic fibers, cystic areas were formed filled with a mucinlike substance. The medial necrosis was most marked in the region of rupture, but was also seen in other parts of the aorta.

The frequency of tear in the first portion of the aorta is probably due to the medial degeneration which occurs with the greatest intensity in this region. With age, there is a tendency for the lesion to become more widely spread throughout the aorta, and it has been suggested by Moritz[31] that the disease is involutional in character.

No definite evidence has been given as to the manner in which the medial necroses occur. Most observers agree that these lesions do not arise because of the disturbance of nutrition due to disease of the vasa vasorum. It appears most likely that these degenerations are due to the action of toxins. These toxins may be bacterial, exogenous, such as nicotine or adrenalin, or meta-

bolic. There is also the likelihood that certain dietary deficiencies may be important factors in the production of this lesion.

SUMMARY

Two cases of dissecting aneurysm are reported.

The aorta in each case showed cystic necrosis of media, without evidence of inflammation.

It is believed that these changes in the media were the underlying process in the formation of the dissecting aneurysms.

The etiology for this degeneration remains obscure, but dietary deficiencies coupled with the action of toxins may be important factors.

REFERENCES

1. Tyson, M. D.: Dissecting Aneurysms, Am. J. Path. **7**: 581, 1931.
2. Babes, V., and Mironescu, T.: Ueber dissezierende Arteriitis und Aneurysma dissecans, Beitr. z. path. Anat. u. z. allg. Path. **48**: 221, 1910.
3. Whitman, R. G., and Stein, H. B.: A Contribution to the Pathogenesis of Dissecting Aneurysms; a Case of Dissecting Mesaortitis (Babes and Mironescu) Without Dissecting Aneurysm, J. M. Res. **44**: 579, 1924.
4. Krukenberg, Ernst: Beitrage zur Frage des Aneurysma dissecans, Beitr. z. path. Anat. u. z. allg. Path. **57**: 329, 1920.
5. Walker, C., and Walker, L.: Sudden Detachment of Aortic Intima (So-called Dissecting Aneurysm), Brit. M. J. **2**: 200, 1919.
6. Ames, D., and Townsend, W. G.: Spontaneous Rupture of the Aorta Exclusive of Ruptured Aneurysms; With an Analysis of 50 Cases, Maryland M. J. **37**: 199, 1897.
7. Weiss, Soma: Clinical Course of Spontaneous Dissecting Aneurysm of Aorta, M. Clin. North America **18**: 1117, 1935.
8. Klotz, O., and Simpson, W.: Spontaneous Rupture of Aorta, Am. J. M. Sc. **184**: 455, 1932.
9. Nicholls, F.: Observations Concerning the Body of His Late Majesty, George II, Phil. Tr. Roy. Soc. London **52**: 265, 1762.
10. Boström, E.: Das Geheilte Aneurysma Dissecans, Deutsche Arch. f. klin. Med. **42**: 1, 1888.
11. Busse, O.: Ueber Zerreissungen und traumatische Aneurysmen der Aorta, Virchows Arch. f. path. Anat. **183**: 440, 1906.
12. Oppenheim, F.: Gibt es eine Spontanruptur der gesunden Aorta und wie kommt sie zustande? München med. Wchnschr. **65**: 1234, 1918.
13. Samson, Paul C.: Dissecting Aneurysms of Aorta, Including Traumatic Type, Ann. Int. Med. **5**: 117, 1931.
14. Rindfleisch, E.: Zur Entstehung und Heilung des Aneurysma dissecans Aortae, Virchows Arch. f. path. Anat. **131**: 374, 1893.
15. Marine, D.: Status Lymphaticus, Arch. Path. **5**: 661, 1928.
16. Hamilton, W. F. (Montreal), and Abbott, M. E.: Coarctation of Aorta of Adult Type: Statistical Study and Historical Retrospect of 200 Recorded Cases With Autopsy, of Stenosis or Obliteration of Descending Arch in Subjects Above Age of 2 Years, Am. Heart J. **3**: 381, 1928.
17. Shennan, T.: Dissecting Aneurysms, Medical Research Council, Special Report Series No. 193, His Majesty's Stationery Office, 1934.
18. Shennan, T., and Pirie, J. H. H.: The Etiology of Dissecting Aneurysm, Brit. M. J. **2**: 1287, 1912.
19. Von Rokitansky, C.: Pathologic Anatomy, Syd. Soc., London **4**: 313, 1852.
20. Köster, E.: Ueber die Entstehung der spontanen Aneurysmen u. d. Chronische Mesarteriitis, Berl. klin. Wchnschr. **12**: 322, 1875.
21. Kellogg, F., and Heald, A. H.: Dissecting Aneurysm of Aorta; Report of Case Diagnosed During Life, With Pathologic Study, J. A. M. A. **100**: 1157, 1933.
22. Crane, M. P.: Medical Papers, Christians Birthday Volume, Baltimore, 1936, Waverly Press, p. 142.
23. Lawrence, John H.: Clinical Symptoms and Signs of Dissecting Aneurysm of Aorta, With Report of Case Diagnosed During Life, Internat. Clin. **2**: 122, 1935.
24. Glendy, R. E., Castleman, B., and White, P. D.: Dissecting Aneurysm of Aorta; Clinical and Anatomical Analysis of 19 Cases, 13 Acute With Notes on Differential Diagnosis, Am. Heart J. **13**: 129, 1937.

25. MacCallum, W. G.: Dissecting Aneurysm, Bull. Johns Hopkins Hosp. **20**: 9, 1909.
26. Peacock, T. B.: Report on Cases of Dissecting Aneurysms, Tr. Path. Soc. London **14**: 87, 1863.
27. Moriani, G.: Ueber einen Fall von Aneurysma dissecans Aortae mit besonderer Berücksichtigung frischer Rupturen der Aortenmedia, Virchows Arch. f. path. Anat. **202**: 283, 1910.
28. Gsell, O.: Wandnekrosen der Aorta als selbstandige Erkrankung und ihre beziehung zur spontanruptur, Arch. f. path. Anat. **270**: 1, 1928.
29. Wiesel, J.: Die Erkrankungen Arterieller Gefässe im Verlaufe akuter Infektionen. 3. Teil. Die akute herdförmige Mesarteriitis der Koronarterien und ihre Folgezustände, Ztschr. f. Heilk. **28**: 69, 1907.
30. Erdheim, J.: Medionecrosis aortae idiopathica cystica, Virchows Arch. f. path. Anat. **276**: 187, 1930.
31. Moritz, A. R.: Medionecrosis aortae idiopathica cystica, Am. J. Path. **8**: 717, 1932.

A STANDARDIZED PROCEDURE FOR THE STUDY OF COAGULATION REACTIONS (IN VITRO)*

JOHN H. FERGUSON, M.D., ANN ARBOR, MICH.

OWING to paucity of knowledge concerning the exact physicochemical nature of the reagents, any investigation of coagulation reactions is unduly dependent upon the particular set of conditions under which the individual experimenter operates. Indeed, his results can often be evaluated only by a full consideration of the limitations imposed by these conditions. The particular advantages claimed for a standardized procedure are (1) control of known variables, (2) a point of departure for the study of physicochemical factors, e.g., temperature, pH, dilution, etc., and (3) a sufficient uniformity of the individual reagents to attempt to relate each of them quantitatively to the coagulation time of the test systems. Several years' experience with a variety of techniques has resulted in the elaboration of the following methods, herewith examined for the degree to which they may claim to serve as a *standardized* procedure for a biochemical approach to coagulation problems.

EXPERIMENTAL TECHNIQUE

Cell-Free (Platelet-Free) Plasma.—A medium-sized, preferably fasting, dog is anesthetized by intraperitoneal barbiturate (e.g., 0.7 c.c. per kg. of 5 per cent pentobarbital sodium). The animal, of course, is to be sacrificed. Blood is collected, with negligible tissue damage, via a paraffined cannula in the femoral artery, the first few cubic centimeters being discarded and the remainder received into one-tenth volume of isotonic (3.8 per cent) trisodium citrate in paraffined centrifuge bottles (250 c.c. capacity). Preliminary centrifugalization yields a turbid plasma containing platelets, some white cells, and usually a trace of red cells. More rapid and prolonged recentrifugalization in tubes (50 c.c. capacity) gives a clear plasma, with a certain opalescence varying with the degree of lipemia. In order to remove all traces of platelets, etc., it is

*From the Pharmacological Laboratory, School of Medicine, University of Michigan, Ann Arbor.

Received for publication, May 13, 1938.

recommended that the twice-centrifugalized plasma be submitted to rapid filtration through a Berkefeld (V), or similar, filter. The filtrate is chilled and kept in the icebox.

Prothrombin.—Fresh Berkefeld-filtered plasma is defibrinated by warming cautiously in water bath to 54°-56° C. for two minutes, and then filtering through coarse filter paper. Prothrombin papers are prepared from 5 c.c. quantities of the filtrate by the acetone method of Howell.[1, 16] The method consists simply in precipitation with an equal volume of acetone, immediate collection of the precipitate on a filter paper in a Büchner funnel, rapidly washing with about 20 c.c. of ether, and drying as quickly as possible in an air current (jet or fan). The dry papers may be kept indefinitely and a sufficient supply may be made from a single animal to last for weeks or months. Prothrombin solutions of desired strength are prepared by macerating the cut-up papers for several hours with a suitable volume of distilled water containing a drop or two of 0.5 per cent sodium bicarbonate, and subsequently filtering.

Prothrombin-Poor Plasma.—Both the magnesium hydroxide[13, 25] and aluminum hydroxide[23] adsorption methods have proved efficacious in our experience, and the following technique is selected from a number of empirical variations tested. One hundred cubic centimeters of 10 per cent aluminum chloride are precipitated with one-half volume of 5 per cent ammonia. The precipitate is removed by centrifugalization and resuspended in distilled water. Some three to five repetitions of the washing and recentrifugalization are necessary before the preparation is sufficiently free from traces of ammonia. About 50 c.c. of distilled water are used to make the final suspension of "alumina *gel*." When shaken with 100 to 120 c.c. of Berkefeld plasma at intervals during a two-hour period at icebox temperature, complete, or, more often, nearly complete, adsorption of the prothrombin is effected without serious loss of fibrinogen. High speed centrifugalization is preferred for recovery of the plasma, although slow filtration through coarse filter paper is often satisfactory. It is important to *restore the pH* of the recovered plasma to approximately 7.5 by adding N/1 hydrochloric acid, drop by drop, with continuous stirring, until a test sample gives the correct color (orange pink) with phenol red indicator.

Prothrombin-Free Fibrinogen.—The fibrinogen is precipitated from the adsorbed plasma by treatment with one-third volume of cold saturated ammonium sulfate, the precipitate being recovered by centrifugalization and redissolved in distilled water, or 0.9 per cent sodium chloride, with the addition of sufficient dilute alkali (sodium bicarbonate) to restore to pH 7.5 (phenol red indicator). It is advisable to filter at this stage and then to repeat the "salting out" once or twice. The fibrinogen solution is the one reagent which must be prepared freshly, since no satisfactory method is known for keeping it in stable form.* The present preparation seldom fails to give a solution (about one-half to three-fourths of the original plasma volume) which is sufficiently stable at icebox temperatures for several days.

*A method based on high-vacuum desiccation at low temperatures is at present under investigation.

Cephalin.—The following method of preparing pure cephalin from brain tissue embodies the original technique of Thudichum[26] and the steps to insure the absence of nonamino nitrogen devised by Levene and Rolf.[19] Freshly hashed brain (hog, calf, sheep), freed, of course, from pia mater, etc., is not dried, but directly extracted with three lots of 95 per cent alcohol followed by two lots of ether. The alcohol and ether extracts are concentrated separately (vacuum distillation—filter pump) and the "protagons" removed from the cooled concentrates before mixing. The mixed concentrates are further evaporated to a viscid watery magma. This is extracted three times with acetone and once with cold absolute alcohol. The residue is exhausted with ether, and the cephalin precipitated from ethereal solution with cold absolute alcohol. This is repeated

TABLE I

CLOTTING PROPERTIES OF EXPERIMENTALLY PREPARED REAGENTS AT VARIOUS STAGES IN COURSE OF PREPARATION. 38° C. PROTOCOL

	CLOTTING TIME
1. Fresh dog blood	2 min.
2. Twice centrifuged citrate plasma + Ca	4 min.
3. Twice centrifuged citrate plasma + Ca + cephalin	3 min.
4. Berkefeld (*V*) filtered plasma (2) + Ca	9 min.
5. Berkefeld (*V*) filtered plasma (2) + Ca + cephalin	1¼ min.
6. $Mg(OH)_2$ adsorbed plasma (4) + Ca	No clot
7. $Mg(OH)_2$ adsorbed plasma (4) + Ca + cephalin	45 min.
8. Fibrinogen from (6) (first precipitation with $(NH_4)_2SO_4$) + Ca	No clot
9. Fibrinogen from (6) (first precipitation with $(NH_4)_2SO_4$) + Ca + cephalin	1½ hrs.
10. Fibrinogen from (8) (second precipitation with $(NH_4)_2SO_4$) + Ca	No clot
11. Fibrinogen from (8) (second precipitation with $(NH_4)_2SO_4$) + Ca + cephalin	Trace (18 hrs.)
12. Fibrinogen from (10) (third precipitation with $(NH_4)_2SO_4$) + Ca	No clot
13. Fibrinogen from (10) (third precipitation with $(NH_4)_2SO_4$) + Ca + cephalin	No clot
14. Prothrombin-free fibrinogen (12) + prothrombin + cephalin	No clot
15. Prothrombin-free fibrinogen (12) + prothrombin + cephalin + Ca	8½ min.*
16. Prothrombin-free fibrinogen (12) + (5 min. prothrombin-cephalin-Ca)	30 sec.
17. Prothrombin-free fibrinogen (12) + (10 min. prothrombin-Ca)	40 min.
18. B.e.† fibrinogen (12) + (5 min. b.e.† prothrombin-cephalin-Ca)	45 sec.
19. B.e. fibrinogen (12) + (10 min. b.e. prothrombin-Ca)	No clot

*Reagents added simultaneously (see text, under *Dilution effect*). The other thrombic mixtures were incubated for the times shown.

†b.e. = benzene extracted.[8]

three or four times until the ethereal solution remains perfectly clear after overnight cooling to 0° C., denoting freedom from cerebrosides. The final alcoholic deposit is a snowy-white material, which has been kept *under absolute alcohol* for two years without undergoing oxidation or detectable loss in coagulative function. Cephalin solutions are prepared by evaporating 5 to 10 c.c. of the alcoholic suspension in a tared flask. An air jet has proved more convenient than the water bath. By adding the same number of cubic centimeters of distilled water as there are milligrams of dried cephalin, a convenient 1:1000 stock solution is obtained. Other dilutions maye be made up as required. Aqueous cephalin solutions should be freshly prepared for all quantitative tests.

Routine Control Tests.—1. One cubic centimeter of *fibrinogen* incubated with 0.25 c.c. each of 1:1000 cephalin and N/10 calcium chloride should give no trace of clot in twenty-four hours (38° C.), indicating complete absence of prothrombin.

2. Each batch of *cephalin* solution should be tested with calcium salt and prothrombin-free fibrinogen. We have never observed any coagulant power attributable to the cephalin per se.

3. Fibrinogen should not be coagulated by any *prothrombin* preparation, with or without added cephalin, in the absence of added calcium salts.

The data in the accompanying protocol (Table I) show the modifications of the coagulation reactions at each stage in the experimental procedures.

Thrombin Formation Tests.—Various mixtures of prothrombin, cephalin, and calcium, together with any other agent (e.g., heparin) it is desired to test, are mixed and held at a chosen temperature (e.g., 20° C. v. infra), and 0.5 c.c. samples are removed at intervals. Each sample is added to 1 c.c. of prothrombin-free fibrinogen in a 10 mm. diameter Wassermann tube in a suitable rack, maintained at 38° C. (thermostatically-controlled water bath), and the clotting time (C.T.) noted. Clotting time plotted against the age of the thrombic mixture (timed from the addition of the calcium salt) gives the *activation curve* for the particular thrombic mixture under investigation. If the same prothrombin and fibrinogen preparations (known to be stable—v. infra) are used throughout, the character of the activation curve is a function of (a) the controlled physical conditions and (b) the amounts of the other reagents used.

DISCUSSION

It is immediately obvious from the data in the accompanying tables that *thrombic activity*, as indicated by clotting time, is not a fixed property of any given thrombic mixture, but rather a dynamic process which must be evaluated with reference to the sequence of change. Unless the particular point in the series of values can be specified on exact experimental grounds, no significance can attach to the use of clotting time as a measure of thrombic activity. It becomes necessary, therefore, to determine the conditions under which the activation curve may be regarded as standardized.

The first consideration relates to the purity and stability of the reagents used. The second factor is the set of physico-chemical conditions, including, for instance, pH, temperature, and dilution variables. The present communication represents a critical examination of these technical problems with a view to proceeding ultimately to an examination of the third variable, viz., quantitative differences relating to each of the specific factors involved.

Purity of Reagents.—The ionizable *calcium* salt[7, 9, 22] and the *cephalin* are chemically pure. Our phospholipid was free from traces of choline and analyzed, almost exactly, N:P::1:1, all the N being in NH_2 form. Its calcium compound[27] did not precipitate, but remained in colloidal solution, with the accompanying acid shift in pH readily demonstrated by bromthymol blue indicator.

Prothrombin (fibrinogen-free) is, unavoidably, a crude protein (or its degradation products), undoubtedly of plasma origin. In an experience including over fifty preparations we have on only one occasion (and that to a very slight

We are indebted to Dr. Quick of Marquette University for a supply of his crude brain extract ("thromboplastin"[24]). On simple recalcification it clotted prothrombin-free fibrinogen in about seven minutes, showing inadequate purification from coagulant precursors.

extent) observed any "spontaneous" (i.e., unexplained) activation. It may be concluded that *added* calcium is essential for its conversion into thrombin. The anomalous data in the literature[1, 15, 16, 20] need searching re-investigation of this point. Owing to such "spontaneous" activation, doubt attaches to the claims of Howell and his pupils (op. cit.) that prothrombin may be converted into thrombin by such agents as alcohol, chloroform, etc., a finding we have been unable to confirm. Using one-tenth volume of 0.005 per cent of Northrop and Kunitz's crystalline trypsin (kindly supplied by the Rockefeller workers) we have confirmed Eagle's[4] demonstration of coagulant activity in trypsinized prothrombin solutions (without added calcium or cephalin). There are, however, sufficient minor differences from the usual thrombin system to warrant doubt that the activation is the same phenomenon in both cases.

Calcium alone always develops a relatively weak coagulant. Cephalin enormously improves the thrombic activity and earlier data[5, 8, 21] lead us to the conclusion that calcium alone is insufficient if sources of "available" phospholipid can be excluded. The Howell prothrombin preparation is usually "poor in available cephalin" and the practicability of working with our preparations even in quantitative studies on the cephalin factor is borne out by the fact that significant clotting time differences (at the minimal zone) accompany variations amounting to but 0.0005 mg. of *added* cephalin per 5 c.c. of prothrombin solution. It is not recommended to attempt further control of the cephalin factor by any routine use of lipid solvents.

Fibrinogen.—On similar grounds to those discussed in connection with prothrombin, we may concede that fibrinogen is a plasma protein fraction free from "significant" calcium and containing only a small amount of "available" phospholipid.[8] It is prothrombin free.

Antithrombic Factors.—The need for a considerable amount (of the order of one in several hundreds) of cephalin to neutralize *minimal* (one in several thousands) quantities of added heparin is recorded in another report.[10] Since one part of cephalin in several millions is quite active in our system, there cannot possibly be any significant antithrombic factor content.

Stability of Reagents.—The calcium chloride solutions require no comment. The keeping properties of *cephalin* solutions have been studied. A 1:1,000 solution, which remains clear and odorless, may be kept in the icebox for a week without demonstrable weakening of its clot-aiding powers. Weaker solutions deteriorate more rapidly.

We have previously noted[9] the excellent stability of our *prothrombin* preparations, but it is advisable to run controls before and after any extended series of quantitative tests, especially with diluted solutions. The *fibrinogen* solutions are highly satisfactory as a rule, but some denaturation may occur during the preparation despite every technical care. Close adherence to the cited method of preparation is recommended. Dialysis is unnecessary and undesirable.[25] Denaturation phenomena in relation to clotting have been considered by Fischer[11, 12] and Wöhlisch.[28] A cool temperature is an important means of controlling denaturation of fibrinogen.

Physical Variables. A. *pH.*—It has long been recognized that clotting can occur over a considerable pH range (5-8, Kugelmass[18]) with a poorly defined optimum close to neutrality. There is no pH change attributable to coagulation.[3, 6] We have made some pH studies (glass electrode and indicator dye methods) with a view to controlling the influence of pH on the stability of our materials at various stages of preparation. The free use of phenol red on test samples and the addition of dilute alkali to *prevent* any tendency toward acidification offer the most practical method of control. The major "acidifying" factors appear to be (1) electrolyte effects on fibrinogen during "salting-out" and (2) the free acidity and buffering of cephalin when present in relatively large amounts (especially in the presence of calcium salts[27]).

B. *Dilution in Relation to the Process of Thrombin Formation (first phase of clotting).*—In tests 15 and 16 of Table I it is noted that the clotting time is much longer when the thrombin-forming reagents are added separately to the fibrinogen than when they are permitted to interact for several minutes prior to the clotting test. This is only partly accounted for by the activation period of the thrombin, which is demonstrated in the thrombin formation curves. A thrombic mixture added to fibrinogen at or near the commencement of activation does not proceed to the completion of clotting at anything like the normal rate (in the corresponding activation curves) notwithstanding the fact that we intentionally omit any citration[2] or similar method theoretically desirable to prevent the activation from continuing in the thrombin-fibrinogen mixture. The experimental fact enables us to obviate the additional procedures, which earlier data[9] suggest are by no means without complicating effects. The simplest explanation is a dilution effect, and this is substantiated by the data in Table II.

TABLE II

EFFECT OF DILUTION (PER SE) ON PROCESS OF THROMBIN FORMATION
(First Phase of Clotting)

A. Composition of Thrombic Mixtures (T) and of Fibrinogen-Thrombin Mixtures

	PROTHROMBIN	H_2O	1:1000 CEPHALIN	N/10 $CaCl_2$	T	H_2O	FIBRINOGEN
I	0.8 c.c.	4 c.c.	0.1 c.c.	0.1 c.c.	0.5 c.c.	0.0 c.c.	1 c.c.
II	0.8 c.c.	3 c.c.	0.1 c.c.	0.1 c.c.	0.4 c.c.	0.1 c.c.	1 c.c.
III	0.8 c.c.	2 c.c.	0.1 c.c.	0.1 c.c.	0.3 c.c.	0.2 c.c.	1 c.c.
IV	0.8 c.c.	1 c.c.	0.1 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	1 c.c.
V	0.8 c.c.	0 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.4 c.c.	1 c.c.

B. Thrombin Formation Data. Clotting Times (Seconds) at 38° C.

T	DILUTION	C. T. AFTER:	2′	5′	10′	20′	30′	60′ (INCUBATION AT 38° C.)
I	1:5		570″	300″	195″	120″	125″	150″
II	1:4		530″	190″	125″	80″	85″	90″
III	1:3		270″	120″	60″	75″	82″	115″
IV	1:2		250″	85″	47″	45″	60″	105″
V	1:1		135″	50″	35″	45″	52″	120″

Here we are investigating dilution per se, taking care to maintain (a) proportions of thrombic components, (b) equivalent amounts of thrombic mixture, and (c) same final dilution. Although the dilution is varied only between 1:1 and 1:5, there is a marked effect upon the earlier phases of thrombin production. The minor variations in thrombin stability and optimum potency are irrelevant.

It may be inferred from these data that comparative tests covering the thrombin activation curves at various dilutions are essential for even a relative evaluation of the dilution factor. Clotting-time data on whole plasma or similar complex systems must be subject to a considerable uncontrolled dilution variable.

C. Dilution in Relation to the Thrombin-Fibrinogen Interaction (second phase of clotting).—A number of workers (e.g.[2, 12]) have sought to establish a comparison of "relative concentrations" of thrombin on the basis of clotting-time values obtained by dilution methods. Eagle (op. cit.), for instance, used serial dilutions of (supposedly stable) thrombic mixtures to obtain standards of reference for converting clotting-time data into "relative percentage of thrombin."

In the following experiment (Table III) dilution of the thrombin in the thrombin-fibrinogen mixture was obtained in three different ways, viz., (A) the usual dilution of the thrombic mixture, (B) increasing the amount of fibrinogen

TABLE III

EFFECT OF DILUTION (DIFFERENT METHODS) UPON THE THROMBIN (T)-FIBRINOGEN (F) INTERACTION
(Second Phase of Clotting)
Stable, Fully-Activated Thrombin at 15° C. Clotting Times (Seconds) at 38° C.

	AMOUNT OF F (ORIGINAL)	AMOUNT OF T (ORIGINAL)	AMOUNT OF H_2O (DILUENT)	RELATIVE T CONC. (FINAL)	RELATIVE F CONC. (FINAL)	T:F (RATIO)	CLOTTING TIME (38° C.)
1	1 c.c.	1 c.c.	----	1:2	1:2	1:1	4″
	A. Dilution of thrombin						
2	1 c.c.	$\frac{1}{2}$ c.c.	----	1:3	2:3	1:2	8″
3	1 c.c.	$\frac{1}{4}$ c.c.	$\frac{1}{4}$ c.c.	1:6	2:3	1:4	10″
4	1 c.c.	$\frac{1}{8}$ c.c.	$\frac{3}{8}$ c.c.	1:12	2:3	1:8	20″
5	1 c.c.	$\frac{1}{16}$ c.c.	$\frac{7}{16}$ c.c.	1:24	2:3	1:16	30″
6	1 c.c.	$\frac{1}{32}$ c.c.	$15\frac{}{32}$ c.c.	1:48	2:3	1:32	52″
	B. Increasing amounts of fibrinogen						
7	$\frac{1}{2}$ c.c.	$\frac{1}{2}$ c.c.	----	1:2	1:2	1:1	4″
8	1 c.c.	$\frac{1}{2}$ c.c.	----	1:3	2:3	1:2	7″
9	2 c.c.	$\frac{1}{2}$ c.c.	----	1:5	4:5	1:4	10″
10	4 c.c.	$\frac{1}{2}$ c.c.	----	1:9	8:9	1:8	15″
11	8 c.c.	$\frac{1}{2}$ c.c.	----	1:17	16:17	1:16	23″
12	16 c.c.	$\frac{1}{2}$ c.c.	----	1:33	32:33	1:32	45″
	C. Dilution of fibrinogen						
13	1 c.c.	$\frac{1}{2}$ c.c.	----	1:3	2:3	1:2	$7\frac{1}{2}$″
14	1 c.c.	$\frac{1}{2}$ c.c.	1 c.c.	1:5	2:5	1:2	10″
15	1 c.c.	$\frac{1}{2}$ c.c.	3 c.c.	1:9	2:9	1:2	13″
16	1 c.c.	$\frac{1}{2}$ c.c.	7 c.c.	1:17	2:17	1:2	25″
17*	1 c.c.	$\frac{1}{2}$ c.c.	15 c.c.	1:33	2:33	1:2	55″

*Exact time of clotting onset masked by dilution.

solution, and (C) diluting the fibrinogen solution. It is obvious from a comparison of series *C* with either *A* or *B* that the clotting time is not affected by the independent variation of the fibrinogen concentration (and hence of the thrombin : fibrinogen ratio). The three series show a remarkable correspondence, and even the slight differences in series *A* are in accordance with a small discrepancy in the final thrombin dilutions as compared with the other two series. It may be concluded, therefore, that the specific dilution (i.e., the concentration) of thrombin is the primary determinant of the clotting time.

It is worth pointing out that the clotting time is concerned solely with the rate of the thrombin-fibrinogen interaction and not with the amount of fibrin

produced. That is why we always time our clots from the *commencement*. To wait for a solid clot in the presence of a weak thrombin introduces the consideration of *amount* of fibrin formed, and this, of course, is governed by a different set of factors, notable among which are questions related to quantity of thrombin and weight of the clot (Eagle[2]), neither of which can as yet be dignified with a biochemical significance.

D. *Temperature.*—The experiments of Table IV afford a good illustration of the well-known dependence of the rate of thrombin formation upon temperature. Attention is called, however, to the relationship of temperature to the rate of thrombin deterioration ("metathrombin"? formation). In the series *A*, the incubation of the thrombic mixture was continued at 38° C., with the evident result that the potency of the thrombin fell off quite rapidly after a brief optimal period. In experiment *B*, a portion of the identical mixture was removed after six minutes of incubation and immediately cooled and kept at 15° C. The stabilization of the thrombin by the lowering of the temperature is strikingly demonstrated.

TABLE IV

EFFECT OF TEMPERATURE ON THROMBIN FORMATION AND STABILITY

Thrombic mixture = 10 c.c. prothrombin + 1 c.c. 1:1000 cephalin + 1 c.c. N/10 $CaCl_2$
T:F = 0.5 c.c. : 1.0 c.c.

Clotting times (seconds) at 38° C.

INCUBATION PERIOD =	1′	2′	5′	7′	10′	20′	30′	60′	90	120′
A. 38° C. throughout	hrs.	hrs.	40″	40″	42″	85″	140″	1200″	hrs.	hrs.
B. 38° C. for 8 min. then at 15° C.*	hrs.	hrs.	40″	40″	41″	45″	45″	45″	50″	60″
C. 15° C. throughout	hrs.	hrs.	hrs.	---	210″	75″	30″	30″	30″	30″

*Clotting tests of series *B* performed 1 min. earlier than times listed.

For "standard" conditions it is recommended that a temperature of 15° to 20° C. be employed for the thrombin activation, accepting the inconvenience of some delay in the process of activation, rather than the risk that differences in the activation curves be due to uncontrolled thrombin deterioration instead of to the variable which is being tested. The use of a temperature of 38° C. for the actual clotting test is justified by the speeding up of the clotting time so that there is seldom a long enough period for appreciable "metathrombin" formation. In certain delayed coagulations, however, this possibility may require further investigation.

Another illustration of the importance of temperature is offered in the data of Experiment V, which was devised in order to emphasize the necessity for controlling thrombin stability in evaluating *relative thrombic activity* by any dilution method.

Experiment V.—Thrombic mixture = 15 c.c. prothrombin + 1.5 c.c. 1:1,000 cephalin + 1.5 c.c. N/10 calcium chloride. After five, ten, thirty minutes' incubation, at 38° C., the clotting times of test samples were 30, 55, 210 seconds, respectively. Between the seventh and ninth minutes, dilutions of 1:2, 1:4, 1:8, and 1:16 were made and kept at 38° C. Between the eleventh and sixteenth minutes, these gave clotting times of 78, 145, 240, and 540 seconds, respectively. Between the thirtieth and thirty-fifth minutes, the corresponding tests yielded

clotting times of 175, 225, 360 and 570 seconds, a marked deterioration which would quite invalidate the use of these values as standards of reference for calculating "thrombin percentage" (Eagle op. cit.). Another series of the same dilutions was made up at approximately the same time as the first series. The second group, however, was kept at 15° C. The clotting times were (a) 72, 140, 220, 450 seconds, respectively (ninth to eleventh minutes) and substantially the same, viz., (b) 72, 140, 215, 460 seconds, respectively (thirtieth to thirty-fifth minutes), some twenty minutes later.

It is concluded that the specific influence of thrombin concentration upon clotting time (v. supra) makes it practicable to use a dilution method for converting thrombic activity (clotting time) into relative thrombin values (e.g., percentages), provided that suitable controls are made to assure stability of the thrombin throughout the experimental period. There is, however, no particular value in such additional procedures where the pertinent information can be read directly off the prothrombin activation curves.

CONCLUSIONS

It is evident from the foregoing technical considerations that the standardization of procedures for the biochemical investigation of coagulation reactions can be achieved to a sufficiently satisfactory extent. The manipulations and controls are somewhat tedious and demand considerable care, but the results offer the possibility of a distinct reliance upon even small changes in clotting-time data in elucidating a variety of variables.

SUMMARY

The standardization of coagulation techniques involves the preparation of isolated reagents (e.g., calcium salts, cephalin, prothrombin, and fibrinogen) controlled for "purity" and stability.

Physical variables necessitating investigation and control include pH, temperature, and dilution factors.

It is recommended that a pH slightly on the alkaline side (about 7.5) be maintained by controlling the test systems with phenol red indicator. A temperature not exceeding 15° to 20° C. is necessary to prevent thrombin deterioration. In most cases a temperature of 38° C. is preferred for the actual coagulation tests.

Under conditions of thrombin stability and controlled dilution variables, the clotting-time values become reliable data of reference for the comparative investigation of a large number of experimental variations in the coagulation systems.

REFERENCES

1. Cekada, E. B.: The Preparation and Properties of Prothrombin, Am. J. Physiol. **78**: 512, 1926.
2. Eagle, H.: Studies on Blood Coagulation. I. The Rôle of Prothrombin and of Platelets in the Formation of Thrombin. II. The Formation of Fibrin From Thrombin and Fibrinogen, J. Gen. Physiol. **18**: 531, 547, 1935.
3. Eagle, H., and Baumberger, J. P.: Studies on Blood Coagulation. III. On the Constancy of Hydrogen Ion Concentration During Coagulation of Fibrinogen by Thrombin, J. Gen. Physiol. **18**: 809, 1935.
4. Eagle, H., and Harris, T. N.: Studies on Blood Coagulation. V. The Coagulation of Blood by Proteolytic Enzymes (Trypsin, Papain), J. Gen. Physiol. **20**: 543, 1937.

5. Ferguson, J. H.: Cephalin Content of Prepared Fibrinogen and Prothrombin Solutions, Proc. Soc. Exper. Biol. & Med. **31:** 929, 1934.
6. Ferguson, J. H., and DuBois, D.: Observations on the pH of Clotting and Citrated Blood, J. Lab. & Clin. Med. **21:** 663, 1936.
7. Ferguson, J. H.: The Blood Calcium and the Calcium Factor in Blood Coagulation, Physiol. Rev. **16:** 640, 1936.
8. Ferguson, J. H.: An Experimental Analysis of Coagulant Activation, Am. J. Physiol. **117:** 587, 1936.
9. Ferguson, J. H.: An Intermediary Calcium Complex in Blood Coagulation, Am. J. Physiol. **119:** 755, 1937.
10. Ferguson, J. H.: Modus Operandi of Commercial Heparin, Proc. Soc. Exper. Biol. & Med. **37:** 23, 1937.
11. Fischer, A.: Studies on Coagulation of the Blood, Jap. J. Exper. Med. **13:** 223, 1935.
12. Fischer, A.: Über die Reaktionsmechanismus der Blutgerinnung, Biochem. Ztschr. **259:** 67, 1933.
13. Fuchs, H. J., and Hartmann, E.: Ueber Proteolytische Fermente im Serum. XII. Die Bedeutung des Komplements bei der Blutgerinnung, Ztschr. f. Immunitätsforsch. u. exper. Therap. **58:** 14, 1928.
14. Goddard, C. H.: The Effect of Filtration Through a Berkefeld Filter Upon the Coagulability of Oxalated Plasma, Am. J. Physiol. **35:** 333, 1914.
15. Howell, W. M.: The Preparation and Properties of Thrombin, Together With Observations on Antithrombin and Prothrombin, Am. J. Physiol. **26:** 453, 1910.
16. Howell, W. H.: Prothrombin, Am. J. Physiol. **35:** 474, 1914.
17. Howell, W. H.: Theories of Blood Coagulation, Physiol. Rev. **15:** 435, 1935.
18. Kugelmass, I. N.: Contribution Physico-Chémique au Mécanisme de la Coagulation du Sang, Arch. internat. de physiol. **21:** 139, 1923.
19. Levene, P. A., and Rolf, I. P.: A Note on the Preparation of Cephalin, J. Biol. Chem. **74:** 713, 1927.
20. Mellanby, J.: Prothrombase—Its Preparation and Properties, Proc. Roy. Soc. S. B., London **107:** 271, 1930.
21. Mills, C. A.: Is Cephalin Necessary in the Activation of Prothrombin? Chinese J. Physiol. **1:** 435, 1927.
22. Nordbö, R.: Über die Aktivitätskoeffizienten der Kalziumionen und der Oxalationen im Plasma und über die Bedeutung der Kalziumionenkonzentration für die Gerinnung des Blutes, Skandinav. Arch. f. Physiol. **75:** Suppl. 11, 1936.
23. Quick, A. J.: On the Relationship Between Complement and Prothrombin, J. Immunol. **29:** 87, 1935.
24. Quick, A. J.: On Various Properties of Thromboplastin (Aqueous Tissue Extracts), Am. J. Physiol. **114:** 282, 1936.
25. Smith, H. P., Warner, E. D., and Brinkhous, K. M.: Lung Extract and Blood Clotting, Am. J. Physiol. **107:** 63, 1934.
26. Thudichum, J. L. W.: A Treatise on the Chemical Constitution of Brain, 1884.
27. Wadsworth, A., Maltaner, F., and Maltaner, E.: Studies on the Activity of Cephalin as It Relates to the Coagulative and Complementary Properties of the Blood, J. Immunol. **30:** 417, 1936.
28. Wöhlisch, E.: Die Physiologie und Pathologie der Blutgerinnung, Ergebn. d. Physiol. **28:** 443, 1929.

MULTIPLE PRIMARY MALIGNANT TUMORS*

J. D. Kirshbaum, M.D., M.S., and F. L. Shively, Jr., B.S., B.M.,
Chicago, Ill.

MULTIPLE primary malignant tumors in the same individual are of great interest since they demonstrate that a malignant neoplasm does not infer immunity against a second malignant tumor. The literature contains many reports describing multiple malignant tumors, but in reviewing this literature one finds discrepancies as to the frequency of this combination as well as the criteria applied in determining whether two tumors are independent of each other.

Billroth[1] is credited with having reported the first case of multiple primary tumors in 1869. Fried[2] considered the condition very rare, finding one case in 1,000 post-mortem examinations. Muller,[3] in reviewing 5,012 autopsies over 20 years of age, noted 1,121 tumors; 18, or 1.6 per cent, had more than one cancer. Harrington[4] studied 1,100 cases of carcinoma of the breast and found the second breast in 37 instances the site of carcinoma, either simultaneously or subsequently. Thirteen cases he considered as multiple primary cancers, an incidence of 1.1 per cent. Hansemann[5] found 5 cases out of 1,000 tumor cases. Redlick,[6] quoted by Ewing,[32] reported 14 cases from a series of 1,225 cases. Hanlon,[7] after carefully reviewing the literature, reported 48 cases. Puhr,[8] in a series of 6,718 necropsies from the second Budapest Pathological Institute, found 1,559 tumors; 219, or 14.04 per cent, were multiple growths, of which 5, or 0.21 per cent, were multiple primary malignant neoplasms. Egli,[9] quoted by Murray, in reviewing 966 tumors at the Basel Pathological Institute, found a multiplicity of tumors in 263, or 27.3 per cent, of which there were 20 multiple malignant growths, or 2.07 per cent. Murray[10] reported a series of 4,219 examinations from the Oslo Cancer Institute to have had 32 multiple malignancies, or 1 per cent, of the cases. Harbtz[11] found 16 cases, or 3 per cent in 524 cancer autopsies. McNamara[12] found one case in 364 autopsies.

Single cases by Bosq,[13] Yamagiwa,[14] Luchsinger,[15] Ball and Reynolds,[16] Jolkwer,[17] Perrotte,[18] Winter,[19] White,[20] Bargen and Rankin,[21] are described. Also two cases by New and Childrey,[22] Duschl,[23] and Seecof.[24]

Peirson[25] reported similar tumors in both testes, and reviewed 46 primary bilateral tumors of the testes.

Hurt and Broders[26] found 71 cases of multiple primary malignant neoplasms, or 3.34 per cent, in 2,124 patients at the Mayo Clinic. The majority of these cases were from surgical specimens and not post-mortem material.

*From the Department of Pathology, Cook County Hospital, Dr. Walter Schiller, Director, and the Department of Surgery, Northwestern University.
Received for publication, May 16, 1938.

TABLE I

SUMMARY OF 1411 CASES OF CARCINOMA

LOCATION OF TUMOR	NO. OF TUMORS AS PRIMARY CAUSE OF DEATH	NO. OF INCIDENTAL TUMORS
Respiratory Tract		
1. Oral cavity	33	3
2. Tonsil	7	0
3. Pharynx	14	1
4. Larynx	15	0
5. Lungs	144	0
Gastrointestinal Tract		
1. Esophagus	82	1
2. Stomach	255	18
3. Intestines		
a. Large	151	22
b. Small	8	0
4. Liver		
a. Primary	26	0
b. Bile ducts	13	2
5. Gall bladder	37	2
6. Extra hepatic bile ducts	49	1
Male Sex Organs		
1. Testicle	15	0
2. Prostate	68	22
3. Seminal vesicle	1	0
Female Sex Organs		
1. Urethra	1	0
2. Ovaries	34	1
3. Uterus		
a. Cervix	77	6
b. Corpus	14	1
4. Vagina and vulva	5	0
5. Breast	70	7
Genitourinary Tract		
1. Kidney	43	26
2. Ureters	0	1
3. Urinary bladder	23	2
Endocrine Organs		
1. Pancreas	67	0
2. Pituitary	2	0
3. Thyroid	13	0
4. Adrenal	2	0
5. Thymus	1	0
Miscellaneous		
1. Skin	18	7
Total	1288	123

Owen[27] found 4.7 per cent of 3,000 cases of primary multiple malignant tumors at the Barnard Free Skin and Cancer Hospital.

Warren and Gates[41] made an extensive review of the literature on the subject of multiple primary malignancies up to the year 1932. They found an incidence of 1.84 per cent of multiple primary malignancies in their collected series of 794 cases of carcinoma. In their own series of 1,078 autopsies in which carcinoma was the primary cause of death, 40 cases were encountered in which there were multiple primary carcinomas giving an incidence of 3.17 per cent.

Burke[42] from 1924 to 1935 reported 583 cancers with an incidence of 28.6 per cent in a series of 2,033 autopsies. In this series there were 46 cases of multiple primary carcinoma, with an incidence of 7.8 per cent.

TABLE II

SUMMARY OF 25 CASES*

CASE NO.	AGE	SEX	RACE	ORGAN INVOLVED			METASTASES	
				TUMOR NO. 1	TUMOR NO. 2	TUMOR NO. 3	TUMOR NO. 1	TUMOR NO. 2
1	65	M	C	Stomach	Sigmoid	----	Present	Absent
2	66	M	C	Stomach	Sigmoid	----	Present	Present
3	50	M	W	Stomach	Esophagus	----	Present	Absent
4	81	M	W	Stomach	Prostate	----	Present	Absent
5	75	M	W	Transverse colon	Skin	----	Absent	Absent
6	74	F	W	Sigmoid	Rectum	----	Present	Absent
7	77	M	W	Sigmoid	Rectum	----	Absent	Absent
8	76	M	W	Rectosigmoid	Kidney	----	Absent	Present
9	66	M	W	Colon	Tonsil	----	Present	Present
10	77	M	W	Common duct	Hepatic flexure colon	----	Present	Absent
11	56	M	W	Common duct	Stomach	----	Present	Absent
12	54	F	W	Hepatic duct	Rectum	----	Present	Absent
13	69	F	W	Gall bladder	Rectum	----	Present	Absent
14	57	M	C	Liver	Rectum	----	Absent	Absent
15	53	M	W	Pancreas	Stomach	----	Present	Absent
16	60	F	C	Lung	Uterus	----	Present	Absent
17	68	M	W	Lung	Prostate	----	Present	Absent
18	59	F	W	Lung	Cervix	Eye	Present	Absent
19	69	M	W	Lip	Liver	Prostate	Present	Absent
20	47	F	W	Cervix	Breast	----	Present	Present
21	65	M	W	Kidney	Rectum	----	Present	Absent
22	35	F	W	Adrenal	Colon	----	Present	Present
23	70	M	W	Esophagus	Kidney	----	Absent	Absent
24	52	M	W	Pyriform sinus and aryepiglottic fold	Stomach	----	Absent	Absent
25	60	F	W	Thyroid	Stomach	----	Present	Absent

*Average age—63.3 years; females—white 7, colored 1; males—white 14, colored 3.

The material studied in our paper was selected from 10,870 consecutive autopsies performed at the Cook County Hospital from 1929 to May, 1938, inclusive. There were 1,411, or 12.98 per cent, carcinomas encountered. One thousand two hundred and eighty-eight of the carcinomas were the primary cause of death, while 123 of the carcinomas were an incidental finding at necropsy. Sarcomas and malignant neurogenic tumors were excluded in our group of cases (see Table I). Twenty-five cases presented multiple primary malignant neoplasms, or 1.77 per cent, of the malignant neoplasms. The 25 cases are briefly summarized in Table II.

CASE REPORTS

CASE 1.—A colored male, aged 65 years. *Clinical Diagnosis:* Carcinoma of the stomach. *Anatomic Diagnosis:* (1) Ulcerated, disk-shaped adenocarcinoma of the lesser curvature of the stomach; (2) polyp of the sigmoid colon with malignant transformation. *Metastases:* (1) to the perigastric, peripancreatic, periaortic lymph nodes, liver, perirenal fat tissue, and mucosa of the small intestine.

CASE 2.—A colored male, aged 66 years. *Clinical Diagnosis:* (1) Atrophic cirrhosis; (2) carcinoma of the stomach. *Anatomic Diagnosis:* (1) Ulcerated medullary carcinoma of the fundus of the stomach and perforation into the retrogastric space and formation of a retrogastric abscess; (2) polyp of sigmoid colon with malignant transformation. *Metastases:* (1) to the perigastric, peripancreatic, and mesenteric lymph nodes; (2) to the liver.

CASE 3.—A white male, aged 50 years. *Clinical Diagnosis:* (1) Carcinoma of the esophagus or upper cardia with metastases to the vertebrae and cervical lymph nodes; (2)

myocardial degeneration. *Anatomic Diagnosis:* (1) Adenocarcinoma of the cardiac end of the stomach with stenosis of the cardiac orifice; (2) hornifying, anaplastic, squamous-cell carcinoma of the esophagus. *Metastases:* (1) to the pleura, liver, greater omentum, periaortic lymph nodes, peritoneum, and diaphragm.

CASE 4.—A white male, aged 81 years. *Clinical Diagnosis:* Carcinoma of the stomach with pyloric obstruction. *Anatomic Diagnosis:* (1) Ulcerated mucus-producing adenocarcinoma of the prepyloric portion of the stomach; (2) adenocarcinoma of the prostate gland. *Metastases:* (1) to the perigastric, mesenteric, peribiliary, and peripancreatic lymph nodes, and to the peritoneum.

CASE 5.—A white male, aged 75 years. *Clinical Diagnosis:* Syphilitic heart disease. *Anatomic Diagnosis:* (1) Adenocarcinoma of the transverse colon with moderate stenosis of the lumen; (2) a small basiloma of the nose. *Metastases:* None.

CASE 6.—A white female, aged 74 years. *Clinical Diagnosis:* Acute intestinal obstruction due to a carcinoma. *Anatomic Diagnosis:* (1) Annular constricting adenocarcinoma of the sigmoid colon with partial obstruction of the lumen; (2) plaquelike adenocarcinoma of the rectum. *Metastases:* to the liver.

CASE 7.—A white male, aged 77 years. *Clinical Diagnosis:* Carcinoma of the sigmoid, causing partial obstruction. *Anatomic Diagnosis:* (1) Stenosing carcinoma at the junction of the descending and sigmoid colon; (2) papilloma of the rectum with carcinomatous transformation. *Metastases:* None.

CASE 8.—A white male, aged 76 years. *Clinical Diagnosis:* (1) Carcinoma of the rectosigmoid junction with metastases to the liver; (2) generalized arteriosclerosis; (3) myocardial sclerosis with mild decompensation. *Anatomic Diagnosis:* (1) Ulcerating, fungating, adenocarcinoma of the rectosigmoid junction; (2) hypernephroid carcinoma of the upper pole of the left kidney. *Metastases:* (1) None to the liver; (2) to the kidney capsule.

CASE 9.—A white male, aged 66 years. *Clinical Diagnosis:* Biopsy of tonsil and palate revealed a squamous-cell carcinoma. *Anatomic Diagnosis:* (1) Colloid carcinoma of the sigmoid colon; (2) hornifying epidermoid carcinoma of the left tonsil. *Metastases:* (1) to the liver, sternum, thyroid, and the left adrenal; (2) extension into the soft palate.

CASE 10.—A white male, aged 77 years. *Clinical Diagnosis:* (1) Carcinoma of the intestine with metastases to the liver, or (2) carcinoma of the pancreas. *Anatomic Diagnosis:* (1) Adenocarcinoma of the common duct at the junction with the cystic duct; (2) circular, fungating, mucous carcinoma of the hepatic flexure of the colon. *Metastases:* (1) to the hepatic and biliary lymph nodes, gall bladder, and the liver.

CASE 11.—A white male, aged 56 years. *Clinical Diagnosis:* (1) Carcinoma of the bile ducts or pancreas; (2) Catarrhal jaundice; (3) silent stone in the common duct. *Anatomic Diagnosis:* (1) Adenocarcinoma of the common duct at the junction with the cystic duct occluding the opening of the cystic duct; (2) stenosing, disk-shaped adenocarcinoma of the pyloric portion of the stomach. *Metastases:* (1) to the peribiliary lymph nodes, liver, and the wall of the stomach.

CASE 12.—A white female, aged 54 years. *Clinical Diagnosis:* (1) Carcinoma of the head of the pancreas, or (2) carcinoma of the bile ducts with obstruction. *Anatomic Diagnosis:* (1) Mucus-producing adenocarcinoma of the hepatic ducts completely obliterating the lumen; (2) ulcerating carcinoma of the rectum. *Metastases:* (1) to the liver and the left lower lobe of the lung.

CASE 13.—A white female, aged 69 years. *Clinical Diagnosis:* Gastrointestinal malignancy. *Anatomic Diagnosis:* (1) Adenocarcinoma of the gall bladder; (2) multiple (2) polyps of the rectum with malignant transformation of one of them. *Metastases:* (1) to the liver and peribiliary lymph nodes.

CASE 14.—A colored male, aged 57 years. *Clinical Diagnosis:* (1) Syphilitic aortic insufficiency; (2) carcinoma of the liver, probably secondary to the stomach with ascites. *Anatomic Diagnosis:* (1) Carcinoma of the liver; (2) polyp of the transverse colon with carcinomatous transformation. *Metastases:* (1) with tumor invasion and obstruction of the portal vein and secondary thrombosis extending into the superior mesenteric vein.

CASE 15.—A white male, aged 53 years. *Clinical Diagnosis:* Recurrent carcinoma of the stomach with metastases to the portal lymph nodes and compression of the bile ducts. *Anatomic Diagnosis:* (1) Adenocarcinoma of the head of the pancreas with compression of the common bile duct; (2) multiple (2) polyps of the stomach with early malignant transformation of one of them. *Metastases:* (1) to the liver, peripancreatic lymph nodes, and the periaortic lymph nodes; tumor thrombosis of the hepatic and splenic arteries, and to the wall of the gall bladder.

CASE 16.—A white female, aged 60 years. *Clinical Diagnosis:* (1) Carcinoma of the corpus uteri with metastases to the left lung, or (2) carcinoma of the right ovary and a secondary bronchiogenic carcinoma; (3) arteriosclerotic heart disease with auricular fibrillation. *Anatomic Diagnosis:* (1) Medullary adenocarcinoma of the main bronchus of the left upper pulmonary lobe; (2) fungating adenocarcinoma of the corpus uteri with extension into the cervical canal and a suppurative endocervicitis. *Metastases:* (1) to the left upper pulmonary lobe and to the left pulmonary hilus lymph nodes.

CASE 17.—A white male, aged 68 years. *Clinical Diagnosis:* (1) Malignancy of the lung; (2) bone tumor of the right axilla. *Anatomic Diagnosis:* (1) Very anaplastic, undifferentiated bronchiogenic, squamous-cell carcinoma of the bronchus of the right upper lobe; (2) adenocarcinoma of the prostate. *Metastases:* (1) to all the pulmonary lobes, vertebral body, ribs, adrenals, kidney, myocardium, stomach, and to the skin of the scalp.

CASE 18.—A white female, aged 59 years. *Clinical Diagnosis:* (1) Multiple metastases to lungs, mediastinum, and subcutaneous tissue of the chest, either a carcinoma of the cervix or a sarcoma of the right orbit. *Anatomic Diagnosis:* (1) Medullary, round-cell carcinoma of the middle lobe of the right lung; (2) ulceration and fibrosis of the cervix uteri with obliteration of the external os (radium sterilized carcinoma of the cervix); (3) sarcoma of the right orbit. *Metastases:* (1) to both lungs, pulmonary hilus, peritracheal, posterior mediastinal, peripancreatic, periaortic lymph nodes, right serratus muscle and diaphragm, greater omentum, anterior mediastinum, both adrenals, both kidneys, and under the capsule of the liver.

CASE 19.—A white male, aged 69 years. *Clinical Diagnosis:* (1) Carcinoma of the lip which was diathermized. *Anatomic Diagnosis:* (1) Epidermoid carcinoma of the upper lip; (2) periportal cirrhosis of the liver and early hepatocellular carcinoma of the right lobe; (3) adenocarcinoma of the prostate. *Metastases:* (1) to the submaxillary lymph nodes of both sides.

CASE 20.—A white female, aged 47 years. *Clinical Diagnosis:* A far-advanced carcinoma of the right breast with multiple metastases. *Anatomic Diagnosis:* (1) Ulcerating, hornifying, squamous-cell carcinoma of the cervix; (2) scirrhous ductal carcinoma of the breast. *Metastases:* (1) to the body of the uterus, vaginal wall, rectum, urinary bladder, periaortic and periiliac lymph nodes, and to the liver; (2) to the skin, right axillary lymph nodes, left clavicle, pleura, lung, and to the right adrenal.

CASE 21.—A white male, aged 65 years. *Clinical Diagnosis:* Annular constricting adenocarcinoma of the rectum with metastases to the lungs. *Anatomic Diagnosis:* (1) Malignant hypernephroid carcinoma of the left kidney; (2) adenocarcinoma of the rectum. *Metastases:* (1) to the renal vein and pelvis, lungs, and liver.

CASE 22.—A white female, aged 35 years. *Clinical Diagnosis:* (1) A pseudomucinous cystadenoma of the left ovary with malignant transformation, or (2) a malignancy of the

colon. *Anatomic Diagnosis:* (1) Hypernephroid carcinoma of the left adrenal; (2) adenocarcinoma of the colon. *Metastases:* (1) invading the veins of the kidney and the inferior vena cava; metastases to the liver and to the right ovary; (2) to the liver and mesentery.

CASE 23.—A white male, aged 70 years. *Clinical Diagnosis:* Malignant hypernephroma. *Anatomic Diagnosis:* (1) Hornifying squamous-cell carcinoma of the upper one-third of the esophagus with marked constriction; (2) recurrent papillary adenocarcinoma of the right kidney. *Metastases:* None.

CASE 24.—A white male, aged 52 years. *Clinical Diagnosis:* Advanced pulmonary tuberculosis and tuberculous laryngitis. *Anatomic Diagnosis:* (1) Ulcerated squamous-cell carcinoma of the left pyriform sinus involving the aryepiglottic fold; (2) adenocarcinoma of the lesser curvature of the stomach.

CASE 25.—A white female, aged 60 years. *Clinical Diagnosis:* (1) From an x-ray of the chest and skull, an osteolytic malignant tumor of the parietal bone with metastasis to the lungs was considered. (2) The diagnosis remained uncertain as to the origin of the tumor. *Anatomic Diagnosis:* (1) Adenocarcinoma of the thyroid; (2) adenomyomatosis of the stomach with carcinomatous transformation. *Metastases:* (1) to both lungs, the right parietal bone, sacral bone, and the right peritracheal lymph nodes.

DISCUSSION

The 25 cases, with apparently two independent primary malignant tumors, were selected from a series of 1,411 malignant tumors. The tumors were situated in different organs, usually in different systems, and frequently showed different histologic structures. These criteria are necessary in order to exclude metastases that may simulate primary tumors. Billroth formulated three rules which he thought had to be adhered to in making a diagnosis of multiple primary malignant tumors: (1) each tumor must have a different histologic appearance, (2) the tumors must have different sites of location, and (3) each tumor must produce its own metastases. The above rules may apply to some cases, but they cannot be fulfilled in all cases of multiple primary malignant tumors. Tumors need not necessarily have a different histologic structure in order to be considered independent. This is illustrated by Case 4, which showed an adenocarcinoma of the stomach and an adenocarcinoma of the prostate. A single organ may be the site of two different primary malignant tumors (Jaffé[28] and Renner[29]), either from an embryologic basis or due to irritants. No metastasis may exist from either of the two tumors, i.e., as shown by 5 of the cases.

Goetze[30] offered the following criteria for the diagnosis of multiple primary malignant neoplasms: (1) the tumors must have the macroscopic and microscopic appearance of the usual tumors of the organs involved, (2) the exclusion of metastasis must be certain, and (3) the diagnosis may be confirmed by the character of the individual metastases. The third point was illustrated in only Case 22 of the series; the liver contained metastatic nodes of two distinct different histologic structures.

Wooley,[31] in an attempt to classify multiple primary malignant tumors, listed them into the following groups: (1) multiple tumors may affect the same tissue and produce identical tumors, or growths of different types; (2) affecting both of a pair of organs need not necessarily be of the same histologic type, e.g., both breasts; (3) affecting different regions of the same system need not

necessarily be of the same histologic type, i.e., the genital tract; (4) different systems may be affected by different types of new growths.

It is apparent that there is no standard definition to characterize multiple primary malignant tumors. One must keep in mind that metastases sometimes show considerable differences from the primary tumor. In the metastases metaplastic changes may be present that are not observed in the primary tumor.

Various theories have been offered for the pathogenesis of multiple primary malignant neoplasms. Ewing[32] states that "The rather common occurrence of two or more tumors in different or same organs of the same subject suggests nothing more than the coincidence in several organs of the general biological factors in the genesis of tumors." He suggests that when minute search is carefully conducted and all forms of tumor growths included, the proportion of multiple primary tumors will be much greater. That there may be an endogenous cause, i.e., the abnormal distribution of chromosomes in nuclei, has been often emphasized.[5] Anomalies in cell division have been repeatedly observed.[33, 34] There may be a mechanical developmental anomaly,[35] or multiple foci of irritation. Toxins may persistently act in the same individual in two different locations.[36] Hadser[37] suggested that there may be multiple foci of embryonal displaced or superfluous cells as the cause. It was thought that individuals with one tumor may exert an immunity against a second tumor. This belief was based on the experimental work of Ehrlich and Apolant,[38] who showed that a transplanted malignant growth in mice conferred immunity against a second inoculation of the same or another tumor. Later workers have shown that this immunity diminishes after a short period. An individual with a malignant growth usually succumbs to it before a second malignant neoplasm develops.

The complex influence of an existing tumor upon neighboring cell groups may predispose to tumor growth.[32] Similar tumors in paired organs may exert different influences in each case. There may be widespread multiple foci of origin which may exhibit neoplastic growth one after the other, thus simulating the lateral diffusion of the tumor process.[39] As an explanation for the involvement of paired organs, Wooley[31] believed that the cells must be in a special state, so that whatever acted upon one organ, whether the active proliferation was irritated by one factor or another, affected similarly its pair. Orr[40] was of the opinion that if accurate observations were recorded of the occurrence of multiple primary malignant tumors, the disputed question of whether one malignant tumor exercises an inhibiting effect on the development of a second would be settled.

The 25 cases described are briefly summarized in Table II. There were 17 males and 8 females. Twenty-one of the cases were white and 4 were colored. The ratio of males to females in our series was 2.2:1, while in Burke's[42] series the ratio was 2:1. There were 7 cases in which the primary tumor showed no metastases; 19 showed metastases. Twenty-one cases of the incidental tumors found at autopsy showed no metastases; 5 showed metastases. Two of the patients were under 50 years of age, 7 were between 50 and 59 years, 9 were between 60 and 69 years, and 7 were over 70 years; an average of 63.3 years.

The youngest male was 50 years, the oldest was 81 years, making an average of 66.0 years of age. The youngest female was 35 years, the oldest was 74 years, making an average of 57.2 years of age. In Hanlon's[7] series, the average age was 62.6; Owen's[27] cases, 62.1 years; and Hurt and Broders'[26] as low as 50.4 years. The low age incidence given by Hurt and Broders, as compared to the authors' and other reports, may be because the material they studied was chiefly surgical specimens. The diagnosis of malignant tumors removed at operation is usually made earlier than the tumors found in autoptic material.

It is frequently noted that neoplasms of older people are less malignant than those of younger persons, and it appears obvious that the one with the slower growing tumor will live longer and thereby perhaps have a better chance of developing a second tumor. The above explanation may account for the frequent occurrence of multiple neoplasms in older people.

If one can generalize from a single case example, it may be said that the first malignant tumor does not influence or predispose to the formation of a second one, as exemplified in Case 8. In this case the first tumor was removed by radical operation, and four years later the patient succumbed to a second primary malignant tumor. There were no evidences of any recurrence of the first tumor.

The frequency with which multiple primary malignant tumors are encountered in the literature speaks against the possibility of one tumor conferring an immunity toward the development of a second independent neoplasm.

The colon was involved in 14 cases, the stomach in 8, and the kidney in 3. In 21 cases the gastrointestinal tract was the site of one of the two malignant tumors. The kidney and colon were the most frequent combination of organs to be affected.

The diagnosis of multiple primary malignant tumors was considered clinically in only one case. The second tumor was always, with the exception of the aforementioned case, an incidental finding. In many of the cases the symptoms produced by the multiplicity of tumors were confusing and in each case the findings were fitted together so as to conform with the diagnosis of a single malignant tumor. Frequently the confusion of a group of unrelated symptoms will be clarified if one will keep in mind the possibility that a patient may be inflicted with two or more independent unrelated conditions, whether it be two primary malignant tumors or two infectious diseases.

Only 4 of the cases presented metastases from each of the two tumors. This illustrates the dictum that in multiple primary malignancies each tumor need not necessarily produce its own type of metastasis in order to make a diagnosis of multiple primary neoplasms in the same individual. So often one of the tumors is slow growing, whereas the second produces extensive metastases and death. In 6 of the cases, the tumors produced no metastases. Only one of the 25 cases presented identical histologic changes in both primary tumors. The corollary that the microscopic appearance of the tumor usually differs when two different organs are affected is of diagnostic aid and for the most part an essential criterion, but is not always applicable to all cases. In 20 of the cases

here described, organs of two different systems were the sites of primary malignant tumor formation, while in two of them, three different organs were the sites of malignancy.

It is very apparent that there are no uniform steadfast laws that may be laid down to designate the diagnosis of multiple primary malignant tumors in the same individual in all given cases.

CONCLUSION

Twenty-five cases of multiple primary malignant tumors selected from 1,411 malignant tumors are described, an incidence of 1.77 per cent.

The incidence of multiple tumors in surgical specimens is much higher and is given between 3.34 per cent and 4.7 per cent.

The colon was the site of one of the multiple tumors in 13 of the cases, or 52 per cent; the colon and kidney were the most frequent combination involved.

The average age was 63.3 years. Seventeen were males and 8 were females.

Multiple malignant neoplasms as an incidental finding without any immunity or direct relationship of one tumor for another in the same individual, are discussed.

Some individuals may be endowed with a congenital, or acquired, predisposition toward tumor formation. These factors may be considered as explanation for the presence of multiple primary malignant tumors in the same individual.

REFERENCES

1. Billroth, T.: Die Allgemeine Chirurgie Pathologie u. Therapie, Berlin, Reimer, 1889, p. 908.
2. Fried, D. M.: Primary Double Cancer, Arch. Path. **5**: 565, 1928.
3. Muller, R. F.: Über multiple nichtsystematisierte Primäre Carcinome und ihre Häufigkeit, Ztschr. f. Krebsforsch. **31**: 339, 1930.
4. Harrington, S. W.: Carcinoma of the Breast, J. Lancet **50**: 1, 1930.
5. Hansemann, V.: Ztschr. f. Krebsforsch. **1**: 183, 1904.
6. Redlich: Quoted by Ewing.
7. Hanlon, F. R.: Multiple Primary Carcinomas, Am. J. Cancer **15**: 2001, 1931.
8. Puhr, L.: Über die Multiplizität der Geschwülste, Ztschr. f. Krebsforsch. **24**: 38, 1926.
9. Egli: Quoted by Murray.
10. Murray, J. A.: Linacre Lecture on Multiple New Growths, Lancet **2**: 800, 1927.
11. Harbitz, F.: Quoted by McNamara.
12. McNamara, W. L.: Multiple Tumors, J. Lab. & Clin. Med. **19**: 269, 1933.
13. Bosq, P.: Multiple Primary Tumors, Semana Med. **1**: 1748, 1932.
14. Yamagiwa, K., Tsukahra, S., and Morimoto, S.: Experimentelle Studie über die Antikörperbildung gegen das Impfkarzinom bei Maus, Gann. **21**: 1, 1927.
15. Luchsinger, R.: Zur Kasuistik der primäre multiplen malignen Tumoren, Frankfurt. Ztschr. f. Path. **40**: 417, 1930.
16. Ball, R. P., and Reynolds, J. A.: Multiple Primary Malignancies, M. J. & Rec. **126**: 287, 1927.
17. Jolkwer, W. E.: Über Gleichzeitiges Vorkommen multipler Geschwülste differenter Art, Arch. f. klin. Chir. **155**: 142, 1929.
18. Perrotte, G.: Multiple Tumors, Morgagni **70**: 1889, 1928.
19. Winter, H.: Multiple Primäre Blastombildung, Ztschr. f. klin. Med. **122**: 627, 1932.
20. White, G. S.: Double Primary Malignant Tumors of the Colon, Ann. Surg. **98**: 186, 1933.
21. Bargen, J. A., and Rankin, F. W.: Multiple Carcinomata of the Large Intestine, Ann. Surg. **91**: 583, 1930.
22. New, G. B., and Childrey, J. G.: Tumors of the Tonsil and Pharynx, 357 Cases, Arch. Otolaryng. **14**: 699, 1931.

23. Duschl, L.: Über Primäre Multiplizität von Geschwülsten, Deutsche Ztschr. f. Chir. **193**: 77, 1925.
24. Seecof, D. P.: Multiple Primary Malignant Neoplasms, J. Cancer Research **8**: 213, 1924.
25. Peirson, E. L., Jr.: Bilateral Tumors of the Testicle, J. Urol. **28**: 353, 1932.
26. Hurt, H. H., and Broders, A. C.: Multiple Primary Malignant Neoplasms, J. Lab. & Clin. Med. **18**: 765, 1933.
27. Owen, L. J.: Multiple Malignant Neoplasms, J. A. M. A. **76**: 1329, 1921.
28. Jaffé, R. H.: Sarcoma and Carcinoma of the Liver Following Cirrhosis, Arch. Int. Med. **33**: 330, 1924.
29. Renner, M. J.: Primary Malignant Tumors of the Ureter, Surg., Gynec. & Obst. **52**: 793, 1931.
30. Goetze, O.: Bemerkungen über Multiplizität primärer Carcinome, Ztschr. f. Krebsforsch. **13**: 281, 1913.
31. Wooley, P. G.: Notes on Multiple Primary Tumors, Boston M. & S. J. **148**: 1, 1903.
32. Ewing, J.: Neoplastic Disease, ed. 3, Philadelphia, 1928, W. B. Saunders Co., p. 36.
33. Boveri, T.: Zur Frage der Entstehung Maligner Tumoren, Jena, 1914.
34. Preisz, H.: Ueber die Entstehung der Neubildungen im Anschluss an eine Analogie, Ztschr. f. ärztl. Fortbild. **19**: 174, 1922.
35. Albrecht, H.: Über das Karzinosarkom des Uterus, Frankfurt. Ztschr. f. Path. **2**: 191, 1908.
36. Winiwarter, O.: Beitr. z. Statistik d. Karzinome, Stuttgart, 1878.
37. Hauser, D. A.: Quoted by Ewing.
38. Ehrlich, P., and Apolant, H.: Beobachtungen über Maligne Mausetumoren, Berl. klin. Wchnschr. **72**: 871, 1905.
39. Ribbert, H.: Das Carcinom des Menschen, Bonn, 1911, p. 367.
40. Orr, J. W.: Multiple Malignant Neoplasms, J. Path. & Bact. **33**: 283, 1930.
41. Warren, S., and Gates, O.: Multiple Primary Malignant Tumors, Am. J. Cancer **16**: 1358, 1932.
42. Burke, M.: Multiple Primary Cancers, Am. J. Cancer **27**: 316, 1936.

LABORATORY METHODS

THE QUANTITATIVE UNRELIABILITY OF THE NITROPRUSSIDE TEST FOR -SH AND -S.S-*

FREDERICK S. HAMMETT, PH.D., AND SIDNEY S. CHAPMAN, B.S.,
NORTH TRURO, MASS.

THE nitroprusside reaction is widely used for the colorimetric demonstration and estimation of sulfhydryl (-SH) and disulfide (-S.S-) in tissues and the products of their activities.

The test as usually applied consists of first saturating an appropriate quantity of substance or solution with crystalline ammonium sulfate; then making it alkaline with one or two drops of concentrated 27-29 per cent ammonium hydroxide; and finally adding one or two drops of 5 per cent sodium nitroprusside solution. In the presence of -SH a fleeting pink to purplish red color develops.

The color is not given by -S.S-. This must first be reduced to -SH. This is done by exposing the material to two or three drops of 5 per cent potassium cyanide or sodium cyanide for ten minutes; after which the above-described procedure is employed.

Clearly such directions admit of many variations. It is but natural, therefore, that the test has not been quantitatively satisfactory. Although the compounds which give a like color and those which interfere with color development are very well known,[1-3] what effects, if any, the reagents themselves may have on variations in color change and intensity have yet to be established.

Obviously no reliable quantitative method can be devised until these are known. Nor can surety be had even of the relatively simple qualitative reaction. For although a positive reaction can fairly safely be taken as evidence of the presence of the sought-for groups, a negative reaction at borderline concentrations might conceivably be as much due to inhibition from the improper use of some reagent as to the absence of reacting sulfur compounds.

These principles make desirable an inquiry into the effects of the reagents of the nitroprusside test on its reaction with -SH and -S.S-. Such is here reported.

As test material we used from 0.5 c.c. to 1 c.c. of approximately M/10,000 cysteine, cystine, glutathione, and p-thiocresol. These were especially purified for us by the Chemical Division of the Lankenau Hospital Research Institute.

*From the Marine Experimental Station of the Lankenau Hospital Research Institute, North Truro, Mass.
Received for publication, January 21, 1938.

Standard procedure was to keep all reagents except one constant and then observe the changes in intensity and duration of color with changes of the variant in order of use and concentration. The results are as follows:

Sodium Nitroprusside: This is the chromogenic compound. As such it is incomplete. Without alkalinization it produces no sure color. The chromogenicity resides in some chemical grouping produced by the action of hydroxyl ion. The nature of this is as yet unknown.

The nitroprusside is usually used in 5 per cent water solution. This has a high color. When the -SH concentration is low (circa M/100,000 to M/200,000) so that the pink color is faint, the tint is obscured and even obliterated by the tint of the reagent. We have found a 1 per cent solution does away with this difficulty. It also is adequate for full color development with -SH.

The nitroprusside solution turns green in warm bright sunlight, be this direct or indirect. This, of course, masks the reaction with -SH. The addition of 0.1 c.c. concentrated ammonium hydroxide to each 10 c.c. prevents the decomposition. Although the addition of ammonium hydroxide is advocated by Giroud and Bulliard[4] as routine, we prefer plain water solution because ammoniated nitroprusside changes color on standing. This is evidence of undesired change in chemical character of the reagent.

It is emphasized that only freshly prepared nitroprusside solutions should be used. New solutions should be made up at least every hour when the assay is protracted.

Ammonium Hydroxide: Although ammonium hydroxide is the customary alkaline reagent, it seemed worth while to determine if the effect is attributable to OH ion and if NH_4 ion participates.

To this end, from 0.10 c.c. to 0.25 c.c. of M/10 sodium hydroxide, M/10 sodium cyanide, M/50 lithium carbonate, and M/15 disodium phosphate were severally substituted for the concentrated ammonium hydroxide. Color was produced in all cases. This shows that OH ion makes sodium nitroprusside chromogenic for -SH.

In running the trials it was found that the color character differed with the cation. Thus while NH_4 ions gave their characteristic pink, Na ions gave a reddish purple, and Li ions a distinct trend to orange and yellow. It is clear that the NH_4 ion as such is a factor in the determination of the kind of color produced in the test.

The fact that other cations give other tints is important in any attempted quantitative use of the test. An unadjustable mixture of cations in the tissue or solution being tested could give colors which are unmatchable with that produced by ammonium hydroxide with a standard.

Studies were made of increasing ammonium hydroxide concentration to see if color intensity is related thereto. It was found that as ammonium hydroxide increased from 1.6 to 4.0 M the color also increased; but that from 8.0 M on, the color tended to fade out. Similar changes occurred in similar trials with the other OH ion sources. Clearly there is an optimum concentration for maximum color production. While this can be determined for pure solutions, its estab-

lishment in tissues and their extracts is unsure. On this basis alone the colorimetric estimation of -SH in biologic material in terms of a standard solution is at best but an approximation.

Disodium phosphate solutions, particularly those which have stood for some time, act to inhibit color development; even though the pH is that to be expected for the M/15 concentration (viz., 8.04). This suggestion of phosphate interference has been borne out in work with root-tip extracts. Here it was found that extracts buffered with 0.1 c.c. phosphate mixture to 1.5 c.c. solution gave much less color than unbuffered controls. It was not the buffering per se which was responsible, since citrate buffered extracts showed no such inhibition. It is evident that accurate quantitative estimation of -SH by nitroprusside in the presence of any considerable amount of phosphates is impossible. Phosphate buffers should be avoided in any work where -SH is to be determined by nitroprusside.

The chromogenic property of Na-nitroprusside with -SH is dependent upon OH ion action. The most controllable source seems to be ammonium hydroxide, probably because of its low pK (1.8 by 10^{-5}). This is important since development of color intensity is affected by the amount of OH ion present.

Ammonium Sulfate: When this salt is added to the nitroprusside, ammonium hydroxide, -SH mixture the color is intensified. The intensification increases with increase in salt concentration up to saturation. Control of this factor rests in being sure that saturation is attained. The practice of adding saturated solutions is inadequate. The solid salt must be used—not its solution—and there must be an excess thereof. Since the white of the crystals acts to dilute the pink of the positive test, balance must be had between saturation and excess salt. This can be determined in each case. Considerable latitude is allowable. Ammonium sulfate, therefore, of all the reagents used in the nitroprusside test is the one about which the least anxiety need be felt as a disturbing factor in semiquantitative assays.

The color produced fades rapidly. It would seem logical in attempting quantitative work to add the intensifying reagent last rather than first in order that its effect may not be diminished by the interval between beginning and ending of reagent introduction. This has been borne out by experience. We, therefore, add solid ammonium sulfate *after* ammonium hydroxide and sodium nitroprusside and just before comparison of test with standard.

How ammonium sulfate acts is unknown. Since it enhances color intensity with pure compounds in solution, it must participate directly in the chromogenic reaction. In this connection, when sulfate is added to 0.5 c.c. of an ammonium hydroxide, nitroprusside, -SH mixture on a spot plate, it is possible to actually see color concentrating on each little crystal. This is not adsorption, but localized chemical reaction, since simple stirring disseminates the color throughout the mixture.

In view of the fact that denaturation of proteins may act to liberate -SH groups,[7] and in view of the fact that ammonium sulfate is a protein precipitant, it might be that some of the enhancing effect in tissue and tissue extracts is derived from denaturation action as well as from direct influence of the compound

on the test as such. If this is so, opportunity for variable results arises from the possibility that variations in kind and initial state of protein might be factors conditioning the amount of denaturation and hence the number of -SH groups liberated thereby. The matter deserves exploration.

Zinc Acetate: Swift fading of color produced in the nitroprusside reaction with -SH is a bar to exact work. Giroud and Bulliard[4] have proposed the use of 10 per cent zinc acetate as a color fixative, and results therewith have been reported.[5-6] The compound does act to prolong the duration of color and to fix it in tissues. But—and this is important—it also acts to decrease color intensity. Thus, when 0.1 c.c. of a 1 per cent solution of zinc acetate is added to 1.0 c.c. of a solution containing M/10,000 of any of the -SH carrying compounds used here—and the nitroprusside test is applied as hereinafter described—the color developed is never of the same order of magnitude as that of a control of the same initial -SH concentration (without acetate). Instead it is always much less, from 50 to 75 per cent mostly, sometimes even more. The reaction is not helped by varying the concentrations of the reagents.

This same inhibition of color intensity production occurs in tissue work. The color which is produced in tissue treated with zinc acetate and then well rinsed is never as good as that produced in similar tissue not treated; and what is more, color is found much more widely distributed in tissue not treated with acetate than in tissue exposed thereto. Thus, although a positive reaction in tissues treated with zinc acetate may indicate the presence of -SH therein, the absence of a positive reaction is no proof -SH is not there. In other words, preliminary treatment of tissues with zinc acetate may prevent demonstration of the true distribution of -SH therein by nitroprusside. It follows that despite the fact that duration of color is prolonged and color is fixed in the nitroprusside test for -SH by zinc acetate, its inhibitive and uncertain action on color intensity makes its use unsafe, either in qualitative demonstration or attempt at quantitative estimation.

Cyanide: Sodium Potassium: This reagent is supposed to show the presence of -S.S-. It is used to reduce disulfide to sulfhydryl which is then demonstrated by nitroprusside. The assumption is made that the total -SH after reduction is the sum of the -SH present before reduction and that derived from -S.S- by cyanide action.

A negative reaction before and a positive reaction after cyanide is evidence that -S.S- is present and -SH absent. An increase in color intensity after cyanide, as contrasted with that given before, is suggestive but not conclusive as to the presence of -S.S-, unless the difference is large. For when the -SH concentration is low it is essentially impossible to discover whether the intensification derives from reduced -S.S- or from the high alkalinity of the dissociated cyanide. It sometimes happens that the color after reduction is less than that given before. It follows that the total -SH after reduction may not be the sum of the initial -SH and that derived from reduction of -S.S- by cyanide reduction. Indeed, in such cases, it is the sum of what initial -SH is left after standing in the highly alkaline cyanide medium and that formed by -S.S- reduction which has escaped destruction by the alkali. It might be here stated that -SH is destroyed on standing in highly alkaline solutions. We have

found more than once that whereas a root-tip extract would show M/20,000 free -SH, the color produced after six minutes' reduction would show only M/100,000. In such cases it is impossible to tell if -S.S- is present. This destroys all confidence in any reputed value of -S.S- determinations by nitroprusside after cyanide reduction. All the method is good for is to sometimes establish the presence of -S.S-; but this with surety only when either no free -SH is present or when the ratio disulfide/free -SH is high.

It should now be evident that quantitative exactitude is impossible of attainment in the colorimetric estimation of -SH via nitroprusside. On the other hand, a not too satisfactory approximation of the order of magnitude of free -SH in solution can be had therewith if suitable precautions and patience are exercised.

Thus there is first prepared a M/1000 cysteine stock solution. From this a suitable range of standards (e.g., M/10,000 to M/100,000) is prepared by dilution. A short series is set up of 1 c.c. amounts in small test tubes. Then 1 c.c. of the solution to be tested, diluted tissue extract or juice, is put into a similar test tube. To each tube is added 0.1 c.c. 27 to 29 per cent ammonium hydroxide and 0.1 c.c. 1 per cent sodium nitroprusside. After brief shaking to ensure mixing, there is rapidly added to each tube 0.5 gm. crystalline ammonium sulfate with quick shaking, and the test mixture is hastily matched with two standards to find which it is greater and which it is less than. The procedure is repeated with narrowing standard limits until the test range is established. With practice and experience it has been possible to tell whether test is closer to M/10,000 than to M/11,000; to M/50,000 than to M/52,000; to M/100,000 than to M/110,000; to M/200,000 than to M/250,000. That is to say, with quick work one can place the free -SH concentration in such material within 5 per cent of its detectable value in all except the very low limits of visible reaction.

Although this reads well and although the trained observer can get even better than 5 per cent placing with "pure solutions" of SH-carrying compounds, it must not be forgotten that tissue extracts and other similar materials may contain many substances, the action of which on the reagents and on -SH itself is unknown. The estimation is at its best but an approximation. But as an approximation it has its uses.

Absolute values for free -SH in fresh tissue slices are unobtainable. All that can be secured is an approximation of the relative concentrations in different parts of one and the same slice or region being looked at while the test is being applied. A useful procedure for gross work is to cover the tissue with 0.25 c.c. water; add 0.05 c.c. 27 to 29 per cent ammonium hydroxide and 0.05 c.c. 1 per cent sodium nitroprusside; and then underline the tissue with 0.25 gm. crystalline ammonium sulfate. For study under high power the amounts and the technique must be varied to suit the need. The order of introduction of the reagents into the test should, however, be just as described.

SUMMARY AND CONCLUSIONS

In vitro experiments made with the reagents of the nitroprusside test and highly purified SH-yielding compounds show that each reagent offers variability factors. With these stabilized the test has a limited use in demonstrating the

presence of -SH and -S.S- under certain definite restrictions which are described in the text. It gives a useful approximation for free -SH. It has no quantitative value for -S.S- determination in cyanide-reduced material. It has no quantitative value, absolute or relative, in the presence of zinc acetate.

REFERENCES

1. Hopkins, F. G.: An Autooxidizable Constituent of the Cell, Biochem. J. **15**: 286, 1921.
2. Kennaway, E. L., and Hieger, I.: Quantitative Studies of the Nitroprusside Reaction in Normal Tissues and Tumors, Biochem. J. **21**: 751, 1927.
3. Pourbaix, Y., and Kennaway, E. L.: The Liberation From Yeast of Substances Giving the Nitroprusside Reaction, Biochem. J. **22**: 1112, 1928.
4. Giroud, A., and Bulliard, H.: Sur une réaction des substances à fonction sulfhydryl, Bull. Soc. chim. biol. **14**: 278, 1932.
5. Beck, K., and Urack, H.: Ueber die Arnold'sche reaction mit nitroprussidnatrium auf eiweissstoffe sowie über die denaturierung des fleischeiweisses durch konzentrierte harnstofflosung, Ztschr. f. Untersuch. d. Lebensmitt. **65**: 399, 1933.
6. Chalkley, H. W.: The Chemistry of Cell Division. VII. The Distribution of Sulphydryl in Amoeba Proteus in Interkinesis and Mitosis, as Shown by the Nitroprusside Test, Protoplasma **28**: 489, 1937.
7. Mirsky, A. E.: Coagulation of Myosin by Dehydration, J. Gen. Physiol. **20**: 455, 1937.

RELIABILITY OF THE AGGLUTINATION TEST FOR UNDULANT FEVER*

With Special Reference to Brucella Agglutinins in Tuberculous Individuals

Howard J. Shaughnessy, Ph.D., Chicago, Ill., and
Thomas C. Grubb, Ph.D., Baltimore, Md.

DIAGNOSTIC agglutination tests for undulant fever may be interpreted in several ways, since a positive test may indicate an active, latent, or past brucella infection, or a nonspecific reaction resulting from the presence of other infectious diseases.[1, 2] Amoia[3] has recently demonstrated experimentally that the anamnestic phenomenon may account for the finding of brucella agglutinins in the presence of other infectious diseases.

According to Topley and Wilson[4] the sera of normal persons may contain brucella agglutinins in a titer of from 1:50 to 1:100, though the status of a "normal" person regarding exposure to brucella infections is rather precarious in view of the now well-recognized widespread incidence of Bang's disease. The reports of Carpenter, Boak, and Chapman,[5] Evans[6] and Scoville[7] make it evident that a negative brucella agglutination test by no means excludes the possibility of even an active brucella infection. To add to the confusion, various workers are not agreed upon the agglutination titer which should be considered of diagnostic significance.[2] Lack of uniform technique in performing the agglutination test is probably responsible for this disagreement upon what constitutes a significant diagnostic titer.[8]

*From the Division of Laboratories, Illinois State Department of Public Health, Chicago, Illinois, and the University of Maryland School of Pharmacy.

Received for publication, March 14, 1938.

It is thus apparent that the clinical significance of the results of a brucella agglutination test is equivocal. This is particularly unfortunate, since the protean symptoms of undulant fever may be easily confused with those of many other infectious febrile diseases,[9] and in many cases the physician is forced to depend entirely upon a laboratory test, usually the agglutination test, for the final diagnosis.

Our attention was first drawn to the problem of the reliability of the agglutination test for undulant fever some time ago when it was found that a small percentage of blood samples sent to the state health department laboratories from a municipal tuberculosis sanatorium* contained brucella agglutinins† in a high titer. Since, so far as we are aware, no study had been made in this country of the possibility of falsely positive brucella agglutination tests being obtained with the sera of tuberculous individuals, it was decided to investigate this problem. The method employed in this work gave us information regarding the general reliability of both the positive and negative brucella agglutination tests, as well as data on the presence of brucella agglutinins in tuberculous sera.

METHOD OF STUDY

From 1930 to 1935, positive brucella agglutination tests were obtained with the sera of 405 patients submitted to the main laboratories of the Division of Laboratories of the Illinois Department of Public Health. It was believed that if the number of patients who had any evidence of tuberculosis and no symptoms of undulant fever could be determined, the possible serologic relationship of these two diseases or the presence of nonspecific agglutinins might be detected. Accordingly, a carefully worded questionnaire was sent to all physicians who had submitted blood samples giving a positive brucella agglutination test during this five-year period. The information requested in this questionnaire was as follows:

Patient's name, age, sex and race.
Occupation at the time the positive agglutination test was obtained.
Final clinical diagnosis—was it undulant fever? (Answer yes or no.)
Did the patient have typhoid fever, paratyphoid fever, tuberculosis, or tularemia six months before or after the positive brucella agglutination test was found?

It will be noted that we have purposely included several diseases other than tuberculosis in the last question, so that the clinician would gain no clue as to the purpose of this questionnaire. Information was obtained concerning 327 of the 405 patients, the remaining number being unaccounted for through death of the physician, failure to make a clinical follow-up, or failure to return the questionnaire.

Reliability of the Positive Undulant Fever Agglutination Test.—The information obtained from the returned questionnaires is summarized in Table I.

*We are indebted to Dr. M. Pollak, Medical Director of the Peoria Municipal Tuberculosis Sanatorium, for sending us blood samples and data on the physical findings and diagnosis of the patients.

†For the purpose of this report we have assumed that any serum which produces a positive brucella agglutination test contains brucella agglutinins, though we realize that this is not necessarily true, for agglutination of the brucella antigen may take place in the absence of specific brucella agglutinins.

TABLE I

CORRELATION OF CLINICAL DIAGNOSIS WITH RESULTS OF THE POSITIVE AGGLUTINATION TEST*

		Per cent
No. of patients with a positive agglutination test	327	
Positive agglutination test confirmed by clinical findings	266	81.34
No. of patients in whom the diagnosis of undulant fever was uncertain	13	3.97
Positive agglutination test not confirmed by clinical findings	16	4.89
Positive agglutination in patients with typhoid fever	4	1.22
Positive agglutination in patients with tuberculosis	25	7.64
Positive agglutination in uncertain cases of tuberculosis	3	0.90
Positive agglutination in patients with paratyphoid fever	0	
Positive agglutination in patients with tularemia	0	

*In this paper only agglutination tests with a titer of 1:160 or above are included. However, it is our policy to report to the physician a titer of 1:80 as a "doubtful positive" and request another specimen. Tests with titers below 1:80 are reported as "negative."

The table indicates that in slightly over 80 per cent of the cases the clinical diagnosis agreed with the results of the agglutination test. If the physician was uncertain about the diagnosis, he was asked to indicate this fact in the questionnaire. As the table shows, the diagnosis was uncertain in about 4 per cent of the brucella cases and in 0.90 per cent of the tuberculosis cases. The number of these uncertain diagnoses is not large enough to affect any general conclusions which may be drawn from the data.

It should be especially noted that the sera of 25 tuberculous individuals, all from the above-mentioned tuberculosis sanatorium, gave positive brucella agglutination tests, the titers ranging from 1:160 to 1:2560. The possibility that these positive tests were false or nonspecific results will be discussed in one of the following sections. If we assume for the moment that these tuberculous patients also had unrecognized, latent, or past cases of undulant fever, then the reliability of the positive agglutination test becomes 88.50 per cent; if we omit these 21 cases altogether, then the reliability of the positive test becomes 88.98 per cent.

Since the original report of Francis and Evans,[10] it is usually stated in the literature that there may be a cross agglutination between brucella and tularemia agglutinins. It is, therefore, of interest to note that of the 327 sera containing brucella agglutinins, not one was reported to have come from a patient with tularemia, although from 130 to 150 cases of tularemia are reported in this state annually. Only a negligible percentage of persons with typhoid fever and none with paratyphoid fever gave a positive brucella agglutination test. The number of persons having a positive brucella agglutination test, who were reported definitely not to have had brucellosis, was 16, or 4.89 per cent of the total number of cases. It appears, therefore, from our data, that a positive brucella agglutination test may be relied upon to indicate clinical undulant fever in about 81 per cent of the cases; if there is no suspicion of tuberculosis, a positive test is reliable in about 89 per cent of the cases.

Reliability of the Negative Agglutination Test.—Since it is obviously of equal clinical importance to know the reliability of the negative as well as the positive agglutination test for undulant fever, we prepared a suitable questionnaire to obtain information regarding the clinical diagnosis of patients whose sera gave a negative agglutination test. This questionnaire, requesting the following

information, was sent to all physicians who had submitted, during the past six months, sera giving a negative brucella agglutination test.

Patient's name, age, and sex.

Final clinical diagnosis.

When no diagnosis was made, any of the following signs or symptoms that the patient had were to be checked.

Fever (continuous or intermittent), weakness, pains (location), sweating, chills, loss of weight or appetite, nervousness, constipation.

Additional information supplementing the above data was also solicited.

The purpose of this questionnaire was to determine the correlation between the negative agglutination test and the clinical diagnosis. In those cases where the diagnosis was uncertain, it was hoped that by checking any of the most frequently observed signs or symptoms listed by Hardy and co-workers[11] we might be able to detect an unsuspected case of undulant fever. Information regarding the clinical diagnosis of 358 patients was obtained and is summarized in Table II.

TABLE II

CORRELATION OF CLINICAL DIAGNOSIS WITH RESULTS OF THE NEGATIVE AGGLUTINATION TEST

No. of patients with a negative agglutination test	358	
Negative agglutination test confirmed by clinical findings	281	(86.2 per cent)
Negative agglutination test in patients with a clinical diagnosis of undulant fever		
a. Sera obtained less than two weeks after onset	3	
b. Sera obtained later than two weeks after onset	5	
c. Date of onset not given	11	
	19	(5.8 per cent)
No. of patients without a definite clinical diagnosis but with symptoms suggestive of undulant fever	26	(8.0 per cent)

Our results indicate that in at least 86 per cent of the cases a negative agglutination test substantiated the physician's diagnosis. In 5.8 per cent of the cases the physician stated that the patient had undulant fever. It will be noted, however, that in at least three of these cases the blood sample was submitted before agglutinins might have had time to develop. It is also probable that this time factor was not considered in some of the cases where no date of onset was recorded by the physician. Some 8.0 per cent of the patients, for whom the physician could give no definite diagnosis, had symptoms suggestive of undulant fever, but we naturally hesitate to hazard a guess as to how many of these patients were actually suffering from clinical undulant fever. From a general consideration of the requested and supplementary information obtained from these questionnaires, and from our own previous experience, we feel that in our hands the negative agglutination test for undulant fever will agree with the clinical diagnosis in at least 90 per cent of the cases.

Cross Agglutinations.—The serologist performing diagnostic tests is ever mindful of the possibility of cross agglutinations as a source of false positive tests. We considered it of more than academic interest to analyze our data for the purpose of determining just how seriously cross agglutinations affect the reliability of the brucella agglutination test. Our laboratory records show that

out of the 314 sera submitted for typhoid and brucella agglutination tests, 5, or 1.6 per cent, of the sera gave cross reactions. Out of 394 sera submitted for brucella and *B. proteus* X19 agglutination tests, 13, or 3.3 per cent, showed cross agglutinations. Of the 71 sera examined for paratyphoid A and brucella agglutinins, 74 for paratyphoid B and brucella agglutinins, or 14 for tularemia and brucella agglutinins, no cross agglutinations were observed. Thus, out of the 867 sera examined for brucella and other agglutinins only 18, or approximately 2 per cent, showed cross agglutinations. It should be emphasized that even these so-called cross agglutinations might have been true agglutinations due to the presence of past, latent, or active brucella infections in patients with typhoid, paratyphoid fever, etc. In practice where such cross agglutinations occur it is frequently necessary to examine several samples from the patient before determining which agglutinins are of diagnostic significance.

Before discussing the significance of a positive brucella agglutination test in tuberculous individuals, we believe it is important to outline briefly the technique of the brucella agglutination test performed in our laboratories during the period covered by this study.

TECHNIQUE OF AGGLUTINATION TEST

A polyvalent antigen of 5 brucella strains (*B. abortus* No. 456 and *B. melitensis* No. 428 from the National Institute of Health; *B. melitensis* No. 304 and *B. abortus* No. 229 from the University of Illinois; *B. suis* No. 71 from the University of Chicago) is prepared by growing these strains separately on Difco liver infusion agar for forty-eight hours at 37° C. The growth of each strain is washed off the slants with 0.85 per cent sodium chloride, and the turbidity of each suspension is standardized to conform with a No. 3 McFarland barium sulfate nephelometer. Equal parts of each suspension are then mixed and enough formalin added to give a final concentration of 0.05 per cent. In performing the test, 0.25 c.c. of the antigen is added to 0.25 c.c. of the diluted serum (final serum dilutions of 1:40 to 1:1280 are routinely used) in each agglutination tube, and the tubes then incubated four hours in a 37° C. water bath. The tubes are next removed from the water bath and placed in the 37° C. incubator for eighteen to twenty-four hours before reading the tests. All of the agglutination tests reported in this paper were performed by one individual.

Since the finding of falsely positive brucella agglutination tests has frequently been attributed to the use of strains containing a rough antigen, we tested the thermoagglutination of all of our strains according to Burnet's[12] technique. None of the strains used in our brucella agglutination tests showed any evidence of thermoagglutination, which is usually interpreted as indicating the absence of rough antigen. Although it seems to be now generally agreed that "no advantage is obtained through the use of separate or combined antigens" (Huddleson[13]) and that an antigen made of *B. abortus* is satisfactory for detecting agglutinins produced by all three species of brucella, we have continued to use the polyvalent antigen since it has given us such satisfactory results. In this connection it may be of interest to note that in a recent publication by Higginbotham and Heathman[14] they state that "The results support the contention that a polyvalent antigen including some local strains should be employed in the routine serological examination for undulant fever."

BRUCELLA AGGLUTININS IN TUBERCULOUS INDIVIDUALS

So far as we know, there has been no specific study made in this country to determine the possibility of finding falsely positive or nonspecific brucella agglutinins in the sera of tuberculous persons. In the recent report of Stone and Bogen[15] concerning the incidence of undulant fever in three tuberculosis sanatoriums, it was tacitly assumed that a positive agglutination test in the tuberculous inmates indicated an active, latent, or past brucella infection. If the possibility of nonspecific agglutinations had been considered in their work, the discrepancies between the skin tests and the agglutination tests, and the apparently rapid disappearance of brucella agglutinins might not have been so puzzling.

Within recent years several Italian workers have studied the incidence of brucella agglutinins in the sera of tuberculous persons. Magliulo[16] found agglutinins for *B. melitensis* and *B. paramelitensis* in the sera of 30 of the 60 patients examined. He concluded that these results were explained by the fact that the tubercle bacillus and *B. melitensis* and *B. paramelitensis* contained a common group antigen. The validity of Magliulo's conclusions is questionable, since he did not test his brucella strains for the presence of a rough antigen, and sera from many patients with nontuberculous diseases, such as endocarditis, psoriasis, puerperal fever, syphilis, carcinoma, etc., also agglutinated *B. melitensis* in titers of 1:100 to 1:150. Sanfilippo[17] examined the sera of 132 tuberculous patients to detect agglutinins for *B. melitensis* and *B. paramelitensis*. The melitensis strains were agglutinated only by the sera of two patients not showing clinical brucellosis, but many of the sera contained agglutinins for *B. paramelitensis*. One of the paramelitensis strains was agglutinated by 33 of the sera, another by 76, another by 80, and the fourth strain was agglutinated by 103 of the 132 sera. On the other hand, Morellini[18] did not find any agglutinins for *B. melitensis* or *B. abortus* in the sera of 60 open cases of tuberculosis. He believed that the positive results obtained by others were due to the use of old strains for antigens or that there were dual infections of tuberculosis and undulant fever.

The relationship between brucella and tuberculous infections was studied by Sarnowiec[19] through the use of the allergic reaction in guinea pigs, cattle, and human beings. He found that tuberculous animals frequently gave a positive reaction to abortin, but animals infected with *B. abortus* reacted slightly, or not at all, to tuberculin.

The meager and conflicting literature on the problem of brucella agglutinins in tuberculous persons does not permit any general conclusions to be drawn at the present time. It is hoped, however, that this report will offer additional information and stimulate further investigation of the problem in this country.

Between 1930 and 1935, our laboratories performed brucella agglutination tests on blood samples from approximately 650 patients shortly after they were admitted to the municipal tuberculosis sanatorium previously referred to. No brucella agglutinins were found in 629, or 96 per cent, of these sera. The majority of the 25 patients whose sera gave a positive agglutination test on the first examination, gave an entirely negative result when their sera was retested after a short interval. For example, one patient's serum showed a titer of

1:2560 on the first test, and four days later no brucella agglutinins could be demonstrated in the serum of this same patient. Another patient had a titer of 1:1280 on the first test, and seven days later the test was completely negative. These puzzling results were obtained despite meticulous care to maintain a uniform technique through the use of the same antigen, dilutions, incubation, etc. It was noted that in the majority of these agglutinations with tuberculous sera, the agglutination was of the finely granular type.

In order to determine whether or not we would obtain similar results from another tuberculosis sanatorium, samples of blood were obtained from 102 patients at the Chicago Municipal Tuberculosis Sanatorium.* Approximately 9 per cent of these sera gave positive brucella agglutination tests; and the majority of these sera showed a much lower titer or were completely negative when retested seven to ten days later. Eight of the patients whose sera gave a positive brucella agglutination test and 14 patients whose sera gave a negative test were given an intradermal injection of "Brucin."† None of these patients showed a positive skin test. We also obtained sera from 28 patients in another county tuberculosis sanatorium, but none of these gave a positive agglutination test.‡

The milk used at the Peoria institution was certified raw milk. The agglutination tests, however, were performed within a few days after the patients had been admitted, so that even if there were some undetected cows infected with Bang's disease in the herd, it is very improbable that the patients had become infected and owed their agglutinins to this source. Carpenter, Boak, and Chapman[5] reported that brucella agglutinins are not passively transferred in milk, so that the presence of brucella agglutinins in the patients shortly after their admission probably could not be accounted for on the basis of passive transfer through the drinking of milk containing brucella agglutinins. Pasteurized milk is supplied to the patients at the Chicago sanatorium, so that there is also probably no chance for infection through this route in this institution.

The results of our investigation indicate that the sera of a small percentage of tuberculous individuals may show a transitory brucella agglutinin content. We believe that these agglutinations should be considered as false positive or nonspecific results since it is inconceivable that an agglutination titer of 1:2560 or 1:1280 should completely disappear within the course of a few days, if we are dealing with a true brucella infection. The well-known phenomenon of the disappearance of antibodies from the blood stream in the agonal period is not pertinent here. The absence of a positive skin test in those patients tested is considered further evidence that we were dealing with a nonspecific agglutination phenomenon. It is not believed that the hypothecated common group antigen of the tubercle bacillus and the brucella organisms could explain our re-

*We wish to express our indebtedness to Dr. Allan J. Hruby and Dr. H. C. Sweany for supplying us with samples of blood.

†The Brucin was obtained through the courtesy of Dr. I. F. Huddleson, and the skin tests were performed by Dr. M. R. Lichtenstein of the Chicago Municipal Tuberculosis Sanatorium.

‡We are indebted to Dr. D. O. N. Linberg, Medical Director of the Macon County Tuberculosis Sanatorium for sending us these sera.

sults, for if such were the case, we would not expect the complete disappearance of agglutinins within a short time. It is possible that in those cases where the agglutination titer did not disappear upon repeated testing, the tuberculous patient also had an unrecognized case of latent or past undulant fever contracted before entering the institution. Since a recent survey by the Bureau of Animal Industry of the United States Department of Agriculture* showed that from 20 to 30 per cent of the cattle in Illinois have Bang's disease, and Torrey and Graham[20] isolated brucella organisms from 50 per cent of 62 raw milk samples collected in widely distributed counties in Illinois, it is quite possible that these patients obtained their brucella infection before entering the institutions.

BRUCELLA AGGLUTININS IN TUBERCULOUS GUINEA PIGS

In order to determine if the presence of brucella agglutinins could be demonstrated in animals experimentally infected with tubercle bacilli, the following study was carried out:

The two weeks' growth of a stock strain of the human tubercle bacillus on Petragnani's medium was washed off with saline and ground in a mortar to obtain a homogeneous suspension. Approximately 500,000 tubercle bacilli were injected subcutaneously into the left inguinal region of 25 guinea pigs. Samples of blood were taken from each animal by cardiac puncture before and at intervals of two weeks after inoculation of the tubercle bacilli. It was originally planned to keep the animals that did not die of tuberculosis for a period of three months, but due to the excessive summer heat (105° to 110° F. in the animal quarters), all of the animals died about nine weeks after inoculation. Using the technique previously outlined, the sera from these animals were examined for the presence of brucella agglutinins. All animals were autopsied for gross evidences of tuberculosis, and smears made of the affected organs to detect the presence of tubercle bacilli.

None of the animals showed brucella agglutinins in their sera before injecting the tubercle bacillus, but four showed agglutinins after the inoculation. Table III shows the time and titer of the agglutinins found in these animals.

TABLE III

POSITIVE BRUCELLA AGGLUTINATION TITERS IN SERA OF GUINEA PIGS INOCULATED WITH HUMAN TUBERCLE BACILLI

	TIME INTERVAL AFTER INOCULATION WHEN BLOOD WAS TAKEN			
ANIMAL NO.	2 WEEKS	4 WEEKS	6 WEEKS	8 WEEKS
643	0	1:80	0	0
A-18	1:40	1:160	0	1:640
637	1:40	1:160	0	0
579	1:40	0	0	0

These results lend experimental confirmation to our findings in human beings, for brucella agglutinins were found in the sera of tuberculous guinea pigs, and the agglutination titers were irregular and of a transient nature. It is interesting to note that in seven of the guinea pigs, no gross evidences of tuberculosis were observed, and no tubercle bacilli were found in stained smears of lymph nodes, spleen, liver, and lungs. It is possible that the infection had not advanced far enough in nine weeks to be detected by our methods of examination.

*Report not yet published.

Of the four animals showing brucella agglutinins, only one (A-18) showed the pathologic changes of tuberculosis. Apparently a progressive infection is not necessary to cause the production of nonspecific agglutinins for brucella organisms.

DISCUSSION

Since the agglutination test for undulant fever has not yet had the test of time in this country that the Widal test and other serologic procedures have, the reliability of its results is frequently questioned. However, our study has revealed the fact that a positive test may be relied upon in 81, and perhaps 89, per cent of the cases, and the negative test in about 90 per cent of the cases. This is a high degree of reliability when it is realized that in a recent study on the serologic diagnosis of syphilis conducted by the United States Public Health Service and carried out by many of the country's leading serologists, it was found that positive results were obtained in only 65 to 88 per cent with known syphilitic sera.[21] It may be objected that our method of determining the reliability of the brucella agglutination test is inherently fallacious, since, as we have already pointed out, the physician is frequently forced to base his final diagnosis solely on the laboratory examination of the patient's blood. We do not believe, however, that this criticism is valid, since in the majority of cases the clinical information was obtained from the physician several months, and in most instances several years, after the positive agglutination test was reported. Thus there was usually ample time to detect and diagnose other diseases which might have been mistaken for undulant fever.

Our results indicate that cross agglutinations with serum containing agglutinins for *B. typhosum* or *B. proteus* X19 may be expected in about 4 per cent of the cases, but this does not constitute a serious limitation to the reliability of the test, as subsequent blood samples from the patient in question will usually show an increase in titer of agglutinins for the infectious agent causing the illness.

From our present work it appears that the sera from tuberculous individuals may produce a positive brucella agglutination test at desultory intervals. This finding is supported by the results obtained with experimentally infected guinea pigs.

The phenomenon of nonspecific agglutination by tuberculous sera is not new, for Hull and Henkes[22, 23] reported it to occur with *B. typhosum* and *B. dysenteriae* (Flexner). Hull and associates[24] also found that several members of the paratyphoid group were agglutinated by sera from tuberculous persons. Apparently there is some nonspecific agent in tuberculous serum which, under certain conditions, is capable of causing the agglutination of a variety of bacterial antigens.

SUMMARY

1. In order to determine the reliability of the agglutination test for undulant fever, information concerning the symptoms and clinical diagnosis of patients whose sera had been examined for brucella agglutinins was obtained through questionnaires sent to the attending physicians.

2. The results of the positive agglutination test for brucellosis agreed with the clinicians' diagnosis in 81 to 89 per cent of the cases; the negative agglutination test was substantiated by the clinical diagnosis in about 90 per cent of the cases.

3. An analysis of our data indicated that cross agglutinations were obtained in about 2 per cent of the sera containing agglutinins for *B. typhosum* or *B. proteus* X19. The possibility that these results may not have been due to true cross agglutinations is discussed.

4. Brucella agglutinins were found in approximately 3 per cent of 650 sera from the patients in one municipal tuberculosis sanatorium and in 9 per cent of the 102 sera from the patients in another tuberculosis sanatorium. The transient and irregular behavior of these agglutinations and the absence of positive skin tests indicated that they were probably nonspecific or false reactions.

REFERENCES

1. Hauptman, W.: Beweist ein positives Agglutinationsergebnis das Vorhandensein von Bangscher Krankheit, Med. Klin. **31:** 1174, 1935.
2. Gray, J. D. A.: The Significance of Brucellar Agglutinins in Human Sera, J. Bact. **25:** 415, 1933.
3. Amoia, R.: Observations sur l'agglutination par les "Brucelles," Soc. internaz. di microbiol., Boll. d. sez. ital. **5:** 171, 1933.
4. Topley, W. W. C., and Wilson, G. S.: Principles of Bacteriology and Immunity, Baltimore, 1929, William Wood & Co.
5. Carpenter, C. M., Boak, R., and Chapman, O. D.: The Significance of Brucella Abortus Agglutinins in Human Serum, J. Immunol. **17:** 65, 1929.
6. Evans, A.: Chronic Brucellosis, J. A. M. A. **103:** 665, 1934.
7. Scoville, W. B.: The Prevalence of Mild Brucella Abortus Infections, J. A. M. A. **105:** 1976, 1935.
8. Evans, A.: Laboratory Procedures for the Diagnosis of Undulant (Malta) Fever, Am. J. Pub. Health **17:** 399, 1927.
9. Hasseltine, H. E.: Current Studies of Undulant Fever, Pub. Health Rep. **44:** 1659, 1929.
10. Francis, E., and Evans, A.: Agglutination, Cross-Agglutination, and Agglutinin Absorption in Tularemia, Pub. Health Rep. **41:** 1273, 1926.
11. Hardy, A. V., Jordan, C. F., Borts, I. H., and Hardy, G. C.: Undulant Fever, Nat. Inst. Health Bull. No. 158, 1931.
12. Burnet, E.: La Thermo-agglutination et l'évolution de l'espéce Brucella, Arch. Inst. Pasteur de Tunis **17:** 128, 1928.
13. Huddleson, I. F.: Brucella Infections in Animals and Man, New York, 1934, The Commonwealth Fund.
14. Higginbotham, M., and Heathman, L. S.: Precipitin and Complement-Fixation Reactions of Polysaccharide Extracts of Brucella, J. Infect. Dis. **59:** 30, 1936.
15. Stone, R. V., and Bogen, E.: Studies of Correlated Human and Bovine Brucelliasis, Am. J. Pub. Health **25:** 580, 1935.
16. Magliulo, L.: Siero-reazione di Wright e Tubercolosi, Gior. di batteriol. e immunol. **10:** 284, 1933.
17. Sanfilippo, E.: Potere Agglutinante del Siero dei Tubercolotici sui Germi del Gruppo Brucella, Gior. di batteriol. e immunol. **11:** 1, 1933.
18. Morellini, M.: La Reazione di Wright per il Micrococco di Bruce sul Siero di Sangue dei Tubercolotici, Riforma. med. **49:** 1697, 1933.
19. Sarnowiec, W.: Contribution a l'Etude des Réactions Allergiques Croiseés dans la Tuberculose et la Brucellose, Ann. Inst. Pasteur **53:** 166, 1934.
20. Torrey, J. P., and Graham, R.: Brucella Abortus in Market Milk, Vet. Med. **31:** 61, 1936.
21. Cumming, H. S., Hazen, H. H., Sanford, A. H., Senear, F. E., Simpson, W. M., and Vonderlehr, R. A.: The Evaluation of Serodiagnostic Tests for Syphilis in the United States, Ven. Dis. Inform., Suppl. No. 1, 1935.
22. Hull, T. G., and Henkes, K.: The Widal Test in Tuberculosis, Am. Rev. Tubere. **8:** 266, 1923.
23. Hull, T. G., and Henkes, K.: The Agglutination of the Flexner Dysentery Bacillus by the Blood Serum of Tuberculous Persons, Am. Rev. Tubere. **8:** 272, 1923.
24. Hull, T. G., Henkes, K., and Cassiday, H.: Agglutination Reactions of the Paratyphoid-Dysentery Group in Tuberculosis, Am. Rev. Tubere. **11:** 79, 1925.

THE IRON OF HUMAN BLOOD SERUM*

BURNHAM S. WALKER, M.D., BOSTON, MASS.

IN TEXTBOOKS of physiologic or clinical chemistry it is usually stated, or tacitly assumed, that the blood contains iron in the form of hemoglobin, and in that form only. On this assumption is based the frequent practice of hemoglobin estimation in blood on the basis of the iron content. That this procedure gives a very close approximation to the correct hemoglobin value is attested by the reports of large series of comparative determinations by several competent investigators.[1-4]

Iron has been recognized in the blood in forms other than hemoglobin. The credit for this discovery should probably go to Biernacki,[5] although the work of Erben[6] and of Fowell[7] is more frequently cited. All these investigators were hampered by the lack of adequate analytical methods, and by the current uncertainty as to the iron content of hemoglobin. This latter difficulty was resolved in 1909 by Butterfield;[8] Fowell apparently did not have the benefit of his figures.

Of present-day investigators, Barkan[9] has been outstanding in his contributions to our knowledge of nonhemoglobin iron. He has demonstrated that a portion of the iron in the red disks differs from hemoglobin iron in being "leicht abspaltbar"—easily separated, or labile to dilute mineral acids. Iron in a similarly labile combination is present in plasma or serum. The labile iron is present in quantities independent of the hemoglobin content of the blood sample. About 65 per cent of the labile iron (fraction E) reacts reversibly with oxygen and carbon monoxide; the remainder (fraction E′) does not. In Barkan's most recent publication, he offers evidence that the labile iron consists of:

I. α-Pseudohemoglobin, derived from hemoglobin by rupture of the porphyrin ring (fraction E)
II. α-Pseudomethemoglobin, derived from I by oxidation of the ferrous to ferric iron (fraction E′)
III. Iron liberated from II along with globin and bilirubin by reduction. This iron is liberated into the plasma where it forms a compound with plasma globulin. This fraction forms a major portion of the plasma or serum iron.

Barkan's hypothesis is strongly confirmed by the observed fact that iron and bilirubin are present in normal human plasma in equimolecular proportions. Increase in blood serum iron with bilirubinemia has been independently reported by Locke, Main, and Rosbash.[10]

*From the Evans Memorial for Clinical Research and Preventive Medicine, Boston.
Received for publication, March 23, 1938.

The nonhemoglobin fractions of blood iron dealt with by Warburg and Krebs[11] and by Klumpp[12] may be considered as comparable, if not identical, with the labile iron of Barkan. Klumpp found 2.2 mg. of nonhemoglobin iron as a mean value in whole blood, making up a mean percentage of 5.3 of the total iron. His thesis is that the variability of this nonhemoglobin iron (in both cells and plasma) precludes the possibility of accurate hemoglobin analysis by any iron method. Josephs[13] found somewhat more nonhemoglobin iron (3 mg. per 100 c.c.) in whole blood, and more recently Josephs and Winocur[14] have demonstrated that this nonhemoglobin fraction of iron in the red disks is increased under certain conditions without a simultaneous increase in the labile iron of Barkan. This brings up the possibility of still other fractions of nonhemoglobin iron, which await more definite characterization. Moore[15] has recently summarized the literature on the quantitative determination of the labile iron in whole blood and given the results of his own analyses which show that from 2.4 to nearly 10 per cent of the iron in normal whole blood is "easily split-off," the percentage apparently depending somewhat upon the mineral acid used, and the method of separation.

This study deals with the iron of human blood serum, which consists, according to Barkan's latest formulation, chiefly of ferric iron in combination with serum globulin. This concept agrees with the observed facts that serum iron is not ultrafiltrable unless acidified,[9] is partially removed by precipitation of the proteins,[16] but still gives some of the reactions of inorganic iron.[17] Moore[15] has shown that the amounts of iron in serum and plasma are essentially identical, provided the separation is made within an hour. Longer standing of blood before separation will result in a measurable transfer of iron from cells to plasma or serum.[9] Moore, Doan, and Arrowsmith[18] assign to plasma iron the function of transport: it represents an equilibrium of rates of iron intake, utilization, and excretion.

Several methods have been proposed for the measurement of serum or plasma iron. The possibility of contamination with hemoglobin has occurred to all investigators of this fraction, and several procedures have been used to eliminate it. Bing and Hanzal[19] corrected their values by carrying out a determination of hemoglobin by the quantitative benzidine method of Bing.[20] Moore[15] used a similar procedure. The use of benzidine in this manner is open to the objection that benzidine is not a specific reagent for hemoglobin, but responds to any substance which has a peroxidase action, including iron itself in the ferric state. A solution of ferric pyrophosphate of the same concentration of iron as normal human blood serum (0.1 mg. per 100 c.c.) will give a detectable blue color with the benzidine reagent used by Moore. In the majority of Moore's analyses the correction was of the order of 1 per cent of the total serum iron, which is insignificant considering the error of the iron analysis itself. Other workers have used trichloracetic acid filtrates of serum or plasma. With this method hemoglobin is precipitated and cannot interfere. The objection to the use of trichloracetic acid is that a variable amount of nonhemoglobin iron is removed in the precipitation; this was first noted by Fowweather[16] and confirmed by Moore.[15] In view of the unsatisfactory outcome of attempts to cor-

rect for the very small amounts of hemoglobin possibly present, the figures in this study are not corrected: any specimens showing visible or spectroscopic evidence of hemolysis have been excluded.

The first experimental phase of this study was concerned with the development of a method permitting the examination of sera with accuracy and reasonable speed. The method developed was a modification of that of Fowweather.[16] It differs from the original method in the following respects: serum is used rather than oxalated plasma; the use of platinum is obviated; complete oxidation to the ferric state is assured by the use of persulfate; the color is more stable; the Evelyn photoelectric colorimeter is used for readings.

Procedure for Serum Iron.—Two cubic centimeter samples of unhemolyzed serum are digested in pyrex test tubes (graduated at 20 c.c.) with 1.5 c.c. of concentrated sulfuric acid. A glass bead is adequate to prevent bumping. The digestion may be carried out slowly on a sand bath or rapidly by the direct flame of a microburner. When the mixture is well charred, 30 per cent hydrogen peroxide is added in 0.5 c.c. amounts, repeating as often as necessary, with continued heating, to produce a solution which remains clear and colorless. Usually three additions are required. A control tube containing 1.5 c.c. of concentrated sulfuric acid should be carried along with the others and should receive the same amount of peroxide. When the contents of the tubes is clear and cooled to room temperature, the volume is made up to 20 c.c., and to each tube is added 1 c.c. of saturated potassium persulfate solution and 4 c.c. of 3 N potassium thiocyanate solution. These two solutions are prepared as prescribed by Wong.[21] The colored solutions may be read in an ordinary colorimeter against a standard prepared by using 0.5 c.c. of a standard iron solution containing 0.01 mg. iron per c.c., made up in a similar tube to 20 c.c. volume, and to which identical amounts of persulfate and thiocyanate solutions have been added as to the unknowns. The Evelyn photoelectric colorimeter obviates the difficulty of comparing by eye readings the rather faint colors that are developed. The optimum filter for iron thiocyanate colorimetry would be at 480 mμ.[22] The 520 mμ filter used for creatinin determinations has given entirely reproducible results.

Normal Values for Serum Iron.—Blood sera from 12 medical students were examined. The highest value found was 0.23 mg. iron per 100 c.c. serum, the lowest value 0.04 mg., the mean value 0.13 mg.

Comparison of these values with those of Moore[15] and of other investigators as tabulated in his recent paper shows that there is fairly general agreement in regard to the normal values for human blood serum iron. Our maximum value is somewhat higher than that observed by Moore, agreeing more closely with that of Tompsett;[17] our minimum is very slightly lower than Moore's, and in agreement with Guthmann, Brückner, Ehrenstein, and Wagner.[23] Recently, Jenkins and Thompson,[29] using the thioglycollic acid method, reported much more iron in the plasma than is generally accepted, and also a marked sex difference unconfirmed by other workers. The most extensive report on blood serum iron published to date is that of Heilmeyer and Plötner.[30] They find an average value in males of 0.126 mg. per 100 c.c., which is in agreement with our average value of 0.13 mg. They also observed a sex difference, but it was small and opposite in direction to that reported by Jenkins and Thompson.

Serum Iron in Pernicious Anemia.—The suggestion that the nonhemoglobin iron of the blood serum might be increased in pernicious anemia is an old one. It was first expressed by Erben,[6] later by Fowell.[7] Riecker[25] and Marlow and Taylor[26] investigated the question, but came to conflicting conclusions. Locke, Main, and Rosbash[16] studied 3 cases of pernicious anemia and found high values (not beyond normal limits) which decreased under liver therapy. Moore, Doan, and Arrowsmith[18] tabulated 10 cases of pernicious anemia, of which 5 showed serum iron values above normal. Heilmeyer and Plötner[30] classify pernicious anemia among the hemolytic anemias, reporting 8 cases. Only one untreated case shows a value above their normal range, but all are high in the normal range, and show a decrease in response to liver therapy. The same lowering of serum iron levels following treatment is demonstrated by Moore, Doan, and Arrowsmith; in one case where liver was temporarily discontinued there was a return to a high value.

This present study adds two untreated cases and several treated cases of pernicious anemia.

Mr. McL., aged 46 years, entered the hospital with 36 per cent hemoglobin and 1.6 million red blood cell count; his serum iron was 0.27 mg. the day before liver therapy was begun. Subsequent values are shown in Table I.

C., aged 36 years, was admitted with 27 per cent hemoglobin and 1.01 million red blood cell count; her serum iron on admission was 0.30 mg. Her course is shown also in Table I.

TABLE I

DAY OF TREATMENT	SERUM IRON (MG. PER 100 C.C.)	
	McL.	C.
2	0.28	
5	0.26	
9	0.18	
12	0.22	
13		0.11
15		0.13
16	0.14	
18		0.12
19	0.09	
22	0.10	0.09
29	0.08	
31		0.08
42		0.08
59		0.13
87		0.14

Fifteen serum iron determinations have been made in cases of pernicious anemia under treatment; of these, only two have been above the normal range. In one case, treatment had been obviously inadequate (hemoglobin 49 per cent, red blood cell count 1.9 million). His serum iron level was 0.26 mg. per 100 c.c., dropping to within the normal range after one week of adequate treatment. The other case had the same serum iron level; treatment, previously adequate, had been discontinued. His blood count was not markedly abnormal (hemoglobin 97 per cent, red blood cell count 4.4 million).

In summarizing the observations in pernicious anemia we may note: untreated cases show uniformly high values, often slightly above the normal range;

one week of adequate liver therapy will bring the values down to within normal limits; lapsed or inadequate treatment will result in increased values, sometimes exceeding the normal limit.

Serum Iron in Hypochromic Anemia.—Animal experiments with horses[27] and with rabbits[28] have indicated that hemorrhage will result in depletion of serum or plasma iron. Barkan[9] has repeated some of these experiments and has shown that a decrease in plasma iron may be observed even when the hemoglobin and red blood cell count are not appreciably affected.

Riecker[25] investigated a number of clinical cases of secondary anemia and found low serum iron values. His values in general deviate so widely from the findings of other workers that his evidence is hardly admissible. Locke, Main, and Rosbash[10] report 2 cases, with serum iron values of 0.015 and 0.04 mg., respectively. Moore, Doan, and Arrowsmith[18] report 14 cases of hypochromic anemia, all but 3 with serum iron values below normal. Heilmeyer and Plötner[30] report 7 cases of acute anemia following hemorrhage, and 12 cases with chronic hemorrhage. The serum iron values fall between 0.01 and 0.04 mg. per 100 c.c.

Observations made at the Evans Memorial on patients with hypochromic anemia show that low serum iron values are the rule; values below the low normal limit (0.04 mg.) are uncommon. There is a consistent tendency for increase in serum iron values following successful treatment or cessation of blood loss. The findings in hypochromic anemia are summarized in Table II.

TABLE II

CASE		SERUM IRON MG. PER 100 C.C.	HEMOGLOBIN PER CENT
J.	Hemophilia with rectal bleeding	0.03	26
	Treated with iron and transfusions (three months later)	0.23	91
T.	Anemia associated with diabetes and hypertension	0.03	68
	After seven days' iron therapy	0.08	78
K.	Nutritional anemia: chronic cholecystitis and multiple neuritis	0.03	74
R.	Carcinoma of pharynx	0.03	61
F.	Carcinoma of stomach	0.04	45
W.	Duodenal ulcer, after hemorrhage	0.04	68
	One month later	0.15	104
H.	Ulcerative colitis	0.06	58
B.	Chronic nephritis	0.09	55
R.	Pyonephrosis and nephrolithiasis	0.08	33
O.	Toxemia of pregnancy	0.05	62
	After six weeks of iron therapy	0.08	75
C.	Hematemeses	0.13	58
	One month later	0.22	84

Serum Iron in Other Types of Anemia.—Several cases studied did not permit easy classification, but showed such marked variations from the normal, as regards serum iron, that they deserve recording.

Mr. C., aged 83 years, was admitted November 24, 1936, and discharged December 19. He was followed as an outpatient for a time, was readmitted March 29, and died April 11, 1937. He had an anemia with achlorhydria, which improved slightly under liver therapy, but without reticulocyte response. He was followed for a time after his first discharge, then failed to appear for treatment. After about two months, he returned in much worse condition.

was readmitted, and died after a few days in the hospital. The anatomic diagnosis was "profound anemia (type undetermined in gross)." The bone marrow sections were examined by Dr. Charles F. Branch and by several other pathologists: the opinion was consistently given that there was a toxic depression of bone marrow activity, probably from infection. Although he apparently did not have pernicious anemia, the patient's serum iron figures are similar to those seen in pernicious anemia cases.

TABLE III

SERUM IRON VALUES IN A CASE OF TOXIC DEPRESSION OF BONE MARROW

DATE	HEMOGLOBIN PER CENT	RED BLOOD CELL COUNT (MILLIONS)	SERUM IRON MG. PER 100 C.C.
November 24	36	1.8	
November 30	52	2.1	1.16
December 7	42	2.0	0.42
January 9	49	2.0	0.21
January 19	44	1.9	0.23
January 26	42	2.0	0.19
March 30	24	1.2	0.25

It seems reasonable to believe that the reason for the high serum iron values is the same in this case as in pernicious anemia; adequate iron supply and storage with deficient utilization by the bone marrow. In both situations there is also the possibility of increased blood pigment destruction.

Mrs. G., aged 40 years, was admitted February 1 and discharged April 24, 1937, with a diagnosis of aplastic anemia, for which no definite cause was established. Her anemia was hyperchromic, with free hydrochloric acid present in the gastric contents. She received liver, iron, and several transfusions during her stay in the hospital, and was discharged not greatly improved. From the viewpoint of the behavior of the serum iron her case is somewhat similar to the preceding one. Feb. 3, 1.6 mg. iron per 100 c.c. serum; Feb. 20, 0.35 mg.; Feb. 25, 0.18 mg.; March 1, 0.31 mg.; April 1, 0.27 mg.

Mr. F., aged 54 years, was admitted September 24, 1936. He had a hyperchromic anemia, reticulocytosis, achlorhydria, enlarged liver and spleen, and serologic evidence of syphilis. He was deeply jaundiced. Treatment with iron, liver, and transfusions did not lead to any great improvement. He was discharged November 18, with diagnoses of tertiary syphilis and acquired hemolytic jaundice. His hemoglobin varied between 24 and 32 per cent, his red blood cell count from 0.9 to 1.2 million. Serum iron determinations: September 24, 0.64 mg.; October 7, 0.28 mg.; October 16, 0.21 mg.; October 26, 0.23 mg; October 29, 0.41 mg.; November 9, 0.76 mg.; November 15, 0.54 mg. The excessive iron in his serum might reasonably be attributed to his accelerated rate of blood pigment destruction.

Serum Iron in Erythremia.—Two cases of untreated polycythemia vera were reported by Moore, Doan, and Arrowsmith,[18] both showing low normal levels. We have had opportunity to study two cases; in one case our findings were similar to those cited; in the other, values slightly above normal range (0.35 to 0.26 mg.) were obtained on repeated examination. The available observations are quite obviously inadequate to make any attempt at conclusions.

SUMMARY

1. A simple method for the measurement of the total iron of blood serum is described.

2. The normal range of iron in nonhemolyzed blood serum is from 0.04 to 0.23 mg. per 100 c.c. serum. The average amount is about 0.13 mg.

3. In pernicious anemia the blood serum iron is moderately increased, and returns to normal with successful liver therapy.

4. In hypochromic anemias the values for serum iron are low, but not necessarily below the normal range. Iron therapy increases the serum iron level in these cases.

5. In aplastic and hemolytic anemias the serum iron levels may be elevated.

REFERENCES

1. Sachs, A., Levine, V. E., and Appelsis, A.: Iron in Human Blood, Arch. Int. Med. **52**: 366, 1933.
2. Helmer, O. M., and Emerson, C. P.: The Iron Content of the Whole Blood of Normal Individuals, J. Biol. Chem. **104**: 157, 1934.
3. Haden, R. L.: The Determination of Hemoglobin by the Iron Content Method, J. Lab. & Clin. Med. **19**: 406, 1934.
4. Johnson, M., and Hanke, M. E.: The Iron Content and Oxygen Capacity of Blood, J. Biol. Chem. **114**: 157, 1936.
5. Biernacki, E.: Untersuchungen über die chemische Blutbeschaffenheit bei pathologischen, insbesonders bei anämischen Zuständen, Ztschr. f. klin. Med. **24**: 460, 1894.
6. Erben, F.: Die chemische Zusammensetzung des Blutes bei perniciöser Anämie, Ztschr. f. klin. Med. **40**: 267, 1900.
7. Fowell, P. H. C.: Iron in the Blood, Quart. J. Med. **6**: 179, 1912.
8. Butterfield, E. E.: Über die Lichtextinktion, das Gasbindungsvermögen und den Eisengehalt des Menschlichen Blutfarbstoffs in normalen und krankhaften Zuständen, Ztschr. f. physiol. Chem. **62**: 173, 1909.
9. Barkan, G.: a. Zur Frage der Einwirkung von Verdauungsfermenten auf das Hämoglobineisen, Ztschr. f. physiol. Chem. **148**: 124, 1925.
   b. Über das leicht abspaltbare Bluteisen und sein Verhältnis zum Hämoglobin, Ibid. **171**: 179, 1927.
   c. Die Verteilung des leicht abspaltbaren Eisens zwischen Blutkörperchen und Plasma und sein Verhalten unter experimentellen Bedingungen, Ibid. **171**: 194, 1927.
   d. Über Säurelösliches Eisen im Blutserum, Klin. Wchnschr. **6**: 1615, 1927.
   e. Über den Gehalt des Blutserums am Eisen, Ztschr. f. physiol. Chem. **177**: 205, 1928.
   f. (and Berger, E.) Differenzierung des leicht abspaltbaren Bluteisens auf Grund seiner Reaktion mit Kohlenoxyd und Sauerstoff, Arch. f. exper. Path. u. Pharmakol. **136**: 278, 1928.
   g. Zur Frage des Blutfarbstoffzerfalls durch verdünnte Säuren, Biochem. Ztschr. **224**: 53, 1930.
   h. Über Bestimmungsmethodik und Eigenschaften des "leicht abspaltbaren," Bluteisens, Ztschr. f. physiol. Chem. **216**: 1, 1933.
   i. Über das Verhalten von anorganischem Eisen nach Zusatz zum Blute, Ibid. **216**: 17, 1933.
   j. Die Unterscheidung des "leicht abspaltbaren" Bluteisens vom Hämoglobineisen und vom anorganischen Eisen, Ibid. **221**: 241, 1933.
   k. Neuere Untersuchungen über das Bluteisen, Forsch. u. Fortschr. **10**: 114, 1934.
   l. Verfahren zur Bestimmung des leicht abspaltbaren Bluteisens, Handb. d. biol. Arbeitsmeth. (E. Abderhalden, editor), Abt. V, Teil 8, Seite 1207, 1935.
   m. (with Olesk, J.) Zur Frage der Identität der Blutkatalase mit dem leicht abspaltbaren Bluteisen, Ztschr. f. physiol. Chem. **236**: 197, 1935.
   n. Weitere Untersuchungen über das säurelösliche Plasmaeisen, Ibid. **239**: 97, 1936.
   o. (and Schales, O.) Trennung der Blutkatalase vom "leicht abspaltbaren" Eisen, Ibid. **244**: 81, 1936.
   p. (and Schales, O.) Hämoglobin und "leicht abspaltbares" Eisen bei der Adsorption und bei der Kataphorese, Ibid. **244**: 257, 1936.
   q. Das "leicht abspaltbare" Bluteisen und das Serumeisen, Klin. Wchnschr. **15**: 169, 1936.
   r. Vereinfachtes Verfahren zur Bestimmung des "leicht abspaltbaren" Bluteisens, besonders des Serum- bzw. Plasmaeisens, Ibid. **16**: 300, 1937.
   s. Chemischer Aufbau und physiologische Bedeutung des "leicht abspaltbaren" Bluteisens, Ztschr. f. physiol. Chem. **248**: 96, 1937.
10. Locke, E., Main, E. R., and Rosbash, D. O.: The Copper and Nonhemoglobinous Iron Contents of the Blood Serum in Disease, J. Clin. Investigation **11**: 527, 1932.
11. Warburg, O., and Krebs, H. A.: Über locker gebundenes Kupfer und Eisen im Blutserum, Biochem. Ztschr. **190**: 143, 1927.
12. Klumpp, T. G.: The Variability of Non-hemoglobin Iron, J. Clin. Investigation **14**: 351, 1935.

13. Josephs, H. W.: The Iron of the Blood, Bull. Johns Hopkins Hosp. **50**: 50, 1935.
14. Josephs, H. W., and Winocur, P.: The Occurrence of Nonhaemoglobin Iron in Whole Blood, Ibid. **61**: 75, 1937.
15. Moore, C. V.: Chemical Methods and Normal Values for Plasma Iron and "Easily Split-Off" Blood Iron, J. Clin. Investigation **16**: 613, 1937.
16. Fowweather, F. S.: The Determination of Iron in Blood Plasma, Biochem. J. **28**: 1160, 1934.
17. Tompsett, S. L.: Thiolacetic Acid as a Reagent for the Determination of the Inorganic Iron Content of Certain Biological Materials, Ibid. **28**: 1536, 1934.
18. Moore, C. V., Doan, C. A., and Arrowsmith, W. R.: The Mechanism of Iron Transportation: Its Significance in Iron Utilization in Anemic States of Varied Etiology, J. Clin. Investigation **16**: 627, 1937.
19. Bing, F. C., and Hanzal, R. F.: A Method for the Estimation of Serum Iron, Proc. Soc. Exper. Biol. & Med. **32**: 1013, 1935.
20. Bing, F. C.: Purification of Benzidine, and an Improved Reagent, for Estimating Hemoglobin in Blood, J. Biol. Chem. **95**: 387, 1932.
21. Wong, S. Y.: Colorimetric Determination of Iron and Hemoglobin in Blood, Ibid. **77**: 409, 1928.
22. Evelyn, K. A.: Personal communication.
23. Guthmann, H., Brückner, M. A., Ehrenstein, and Wagner, H.: Das ultrafiltrable Eisen im Serum der Frau, Arch. f. Gynäk. **147**: 469, 1931.
24. Starkenstein, E., and Weden, H.: Über das anorganische Eisen des Organismus, Arch. f. exper. Path. u. Pharmakol. **134**: 274, 1928.
25. Riecker, H. H.: Iron Metabolism in Pernicious and in Secondary Anemia, Arch. Int. Med. **46**: 458, 1930.
26. Marlow, A., and Taylor, F. H. L.: Constancy of Iron in the Blood Plasma and Urine in Health and in Anemia, Arch. Int. Med. **53**: 551, 1934.
27. Fontès, G., and Thivolle, L.: Non-hemoglobin Iron Content of Serum, and Its Diminution in Experimental Anemia, Compt. rend. Soc. de biol. **93**: 687, 1925.
28. Thoenes, F., and Aschaffenburg, R.: Der Eisenstoffwechsel des wachsenden Organismus, Berlin, 1934, S. Karger. (Quoted from Barkan.)
29. Jenkins, C. E., and Thompson, M. L.: The Distribution of Iron in Blood, Brit. J. Exper. Path. **18**: 175, 1937.
30. Heilmeyer, L., and Plötner, K.: Das Serumeisen und die Eisenmangelkrankheit, Jena, 1937, Gustav Fischer.

MODIFIED APPARATUS FOR OBTAINING GASTRIC CONTENTS*

JULES L. POSNER, B.S., JOHN T. MYERS, PH.D., M.D., AND ANDREW R. FODOR, B.A., NEW YORK, N. Y.

SINCE 1812, when it was first used by Philip S. Physick, and in 1822, by Jukes, as a means of relieving the stomach of poisons, the stomach tube has undergone little modification. In 1869, Kussmaul used it clinically for pyloric carcinoma and spasm, first experimenting on a professional sword swallower.

The general structure of the tube has been functionally preserved. Its total length is about 38 inches, of which about 20 to 24 inches extend from the mouth to the stomach. This allows 5 to 8 inches to dip into the stomach. The diameter of the older type tube (Boas) is approximately 12 mm. The distal end tapers to an opening and has two or more smaller openings in the side of the tube, ½ to 1 inch from the tip.

The tube is introduced into the pharynx, and the patient is instructed to close his lips around it and breathe rapidly in order to avoid undue abdominal

*From the Research Department of Bendiner and Schlesinger Laboratories, New York.
Received for publication, March 28, 1938.

stress. The end of the tube is suspended in a flask in which the stomach contents is collected. Cummer, as well as Todd and Sanford, has suggested that a Pollitzer bag be attached to the end of the tube, which is compressed until the esophagus is entered. Release of the bag then causes suction, forcing the stomach contents into the bag.

This process is rather awkward. A single compression is not always sufficient to free a badly clogged tube. The bag must then be removed, recompressed, and attached to the tube as often as necessary to obtain sufficient material. Direct recompression without removal would force the material back through the tube. The bag must be carefully cleaned and sterilized before each case.

The duodenal tube was devised in 1895 by J. C. Hemmeter and later modified by Kuhn, Einhorn, Rehfuss, and others.

The Rehfuss modification is most extensively used both for gastric and duodenal extractions. A small olive-shaped metal tip terminates this tube. Due to its small diameter, 3 to 4 mm., the patient will bear it comfortably, but it becomes easily clogged, making it essential to use suction. This is directly applied with a large aspirating sypringe attached to the tube. The difficulties of this arrangement are obviously similar to those encountered in the use of the Boas tube. In spite of any precautions, the authors have found that mucus and food particles will often clog the tube, necessitating its removal for washing and causing considerable discomfort to the patient. We have attempted to introduce distilled water into the clogged tube to produce syphon action, but results have been too variable.

PROCEDURE

The modified procedure which the authors submit for approval may be used with the Boas or Rehfuss tubes, either for fractional or occasional extraction of gastric contents. This is a modified suction method with the addition of a Florence type flask to serve both as a suction generator and a trap for the extracted material. The flask is tightly fitted with a two-holed rubber stopper. Through one opening is passed a hard rubber nozzle extending slightly into the flask. A piece of hard glass of adequate diameter and of sufficient length to reach from 1 inch above the stopper to about halfway above the bottom of the flask is passed through the other hole.

The end of the gastric tube is attached to the upper end of the glass tube. A rubber bulb with a hard rubber opening to fit tightly over the protruding nozzle is obtained. The apparatus sold by the Davol Rubber Co., as the "Ewald Evacuator," with a rubber bulb of 12 ounces capacity and a glass window in the tube by means of which the flow of material may be observed, has been most effectively used by the authors. This outfit is easily adaptable for this procedure by manipulating as suggested above. The tube is introduced into the stomach, the bulb is compressed, placed on the nozzle of the receiving flask and released. This causes the formation of a slight vacuum, which is usually sufficient to start the flow of gastric fluid into the receiving flask. Food particles or mucus which now clog the tube are freed by slight compression and release

of the bulb which remains fixed on the nozzle. The bulb never comes in direct contact with the extracted material, so that ordinary cleaning and drying is sufficient to prepare it for use (Fig. 1).

When fractional extraction is required, a pinch clamp may be used to close the tube when a sufficient amount of sample has been collected.

The described method has been used by the authors on patients suffering from a variety of disorders, from normal to advanced cases of carcinoma. Particularly successful results have been obtained in aspirating gastric contents from a fasting stomach for bacteriologic examination, especially in connection with the newer technique for diagnosis of pulmonary tuberculosis. We have yet to experience failure in obtaining sufficient substance for examination or to note

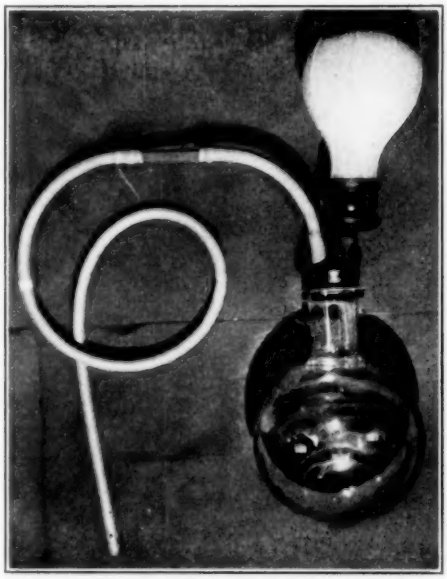

Fig. 1.—Photograph of described apparatus.

any harmful effects of the procedure on the patient. On the contrary, the patient suffers less discomfort than in any of the current procedures, due greatly to the speed with which the whole operation can be accomplished.

SUMMARY

An improved method for the removal of gastric contents having the following advantages is described:

1. Clogging of the tube and repeated washings are made unnecessary because of indirect syphon action.

2. Larger quantities of test meals are recovered, permitting more accurate analyses and interpretation.

3. It permits the removal of gastric material without previous ingestion of test meals.

4. It decreases the discomfort of the patient due to greater speed and certainty of action.

REFERENCES

1. Cummer, C. L.: Manual of Clinical Laboratory Methods, Philadelphia, 1926, Lea & Febiger, p. 319.
2. Hillsman, J. A.: Continuous Gastric Lavage, Canad. M. A. J. **29**: 186, 1933.
3. Physick, P. S.: Account of a New Mode of Extracting Poisonous Substances From the Stomach, Eclectic Repertory, Philadelphia **3**: 111, 1812-13. Ibid. **3**: 380. After submitting this paper for publication it was found that Dr. Physick had later on given credit for the invention to Dr. Alexander Monro of Edinburgh who had published it in his Inaugural Thesis in 1797. This was unknown to Dr. Physick at the time of his original article.
4. Jukes, E.: A Method of Saving Life in Cases of Poisoning, by Means of a Pump, Med. Repertory, London **2**: 112, 1822.
5. Kussmaul, A.: Ueber die Behandlung der Magenveiterung durch eine neue Methode, Deutsches Arch. f. klin. Med. **6**: 385, 1895.
6. Hemmeter, J. C.: An Apparatus for Washing Out the Stomach and Sigmoid, New York M. J. **61**: 385, 1895.

THE DETERMINATION OF MORPHINE IN THE URINE OF MORPHINE ADDICTS*

FRED W. OBERST, M.S., PH.D., LEXINGTON, KY.

A QUANTITATIVE determination of morphine in urine consists of the isolation of the alkaloid in a relatively pure state and the measurement of its concentration. Most of the methods reported in the literature are satisfactory only when the amounts of morphine in urine are relatively high as in cases of acute morphine poisoning or in experimental animals receiving a high morphine intake. The amount of morphine found in the urine of clinical cases and of most morphine addicts is considerably lower, consequently the methods described are not practical, since it becomes necessary to use excessively large volumes of urine to carry out an analysis. Because the question of the fate of morphine is being studied in the addict, it became necessary to evaluate the various methods already described and to develop a more practical and sensitive method. The procedure of Pierce and Plant[1] still is the most satisfactory quantitative method for the determination of morphine in biologic materials. Their extraction procedure for a quantitative removal of morphine from urine has not been surpassed. They removed morphine with an ethyl alcohol-chloroform mixture from 60-70 ml. of urine saturated with sodium bicarbonate using a continuous liquid-liquid extractor. The residue obtained after the evaporation of the solvent was dissolved in 0.5 per cent hydrochloric acid and subjected to three extractions with an amyl alcohol-chloroform mixture to remove impurities. Then they saturated the aqueous solution with powdered sodium bicarbonate and removed the alkaloid by three extractions with an amyl alcohol-chloroform mixture. The extraction solvent containing the morphine was treated with a few drops of dilute hydrochloric acid and evaporated to dryness. The morphine concentration in the final residue, ranging from 1 to 20 mg., was determined

*From the United States Public Health Service Hospital, Lexington.

Read before the Federation of American Societies for Experimental Biology, Baltimore, Md., March 30—April 2, 1938.

colorimetrically by means of the Lautenschläger diazobenzenesulphonic acid method.[2] Their studies were made on dogs receiving doses of morphine ranging from 10 to 50 mg. per kg. body weight; consequently large amounts of the drug were excreted in the urine.

Many other methods for the isolation and determination of morphine are described in the literature, most of the earlier methods are adequately reviewed by Balls and Wolff[3] and Deckert.[4] Most of these methods are strictly qualitative. Balls and Wolff,[3] Fry, Light, Torrance, and Wolff,[5] and Wolff, Riegel, and Fry[6] used a continuous liquid-liquid extractor and made repeated isoelectric extractions until their residue was pure enough for a silicotungstic acid precipitation. The precipitate was removed, dried, and weighed. Their method lacked sensitiveness, at least 5 mg. and better 10 mg. of morphine being required for a reasonably accurate analysis. When the amount of morphine in a single determination was less than 8 mg., the final estimation was made colorimetrically by the diazo reaction.

Loofs[7] in 1922 was one of the first to study successfully the excretion of morphine clinically in human beings. He removed morphine from urine by the classical Stas-Otto procedure of repeated evaporations and acid alkaline extractions. The final residue was tested by one of the well-known alkaloidal color reagents (Fröhde, Marquis, Pellagri). This method required the use of large quantities of urine and detected only the presence of morphine.

Panse[8] tried to simplify the Stas-Otto method and limited the quantity of urine for an analysis to 50-100 ml. He used ethyl acetate to extract the morphine and then sublimed the alkaloid for its detection by the usual alkaloidal color reagents. Teruuchi and Kai,[9] Ellinger and Seeger,[10] To and Rin,[11] Frehden and Huang,[12] Liang[13] and others reported various procedures for extracting morphine from urine. The presence of morphine in the final residue was determined by the usual alkaloidal color or precipitation tests. Teruuchi and Kai determined the concentration by titration according to Gordin's method.

Deckert[4] described a method suitable for clinical use in which morphine was extracted from urine by means of ethyl acetate and was determined nephelometrically as a morphine-molybdate-vanadate complex, the turbidity produced being roughly proportional to the amount of morphine present. He claimed that his method is rapid and sensitive, and that he obtained a satisfactory morphine test on 10 ml. urine containing as low as 0.01 mg. morphine.

With the exception of the Deckert method it is apparent that the methods for the quantitative determination of morphine in a single extraction from urine are not satisfactory for amounts below 1 mg. In the following report methods and procedures are presented for the quantitative determination of 0.03 to 2.0 mg. of morphine in 10 to 70 ml. urine. A new method is described for the further separation of morphine from impurities in a urinary residue by means of permutit. The morphine concentration in the permutit is then determined colorimetrically by a new procedure for morphine, which is more sensitive than previous colorimetric methods. The Deckert method has been improved and made more reliable for the detection and determination of small amounts of morphine.

EXPERIMENTAL

Reagents.—Sodium bicarbonate, U.S.P., powdered.

Ethyl alcohol, 95 per cent, U.S.P., is treated with charcoal (Norit), filtered, redistilled on a water bath, leaving about 10 per cent in the distilling flask.

Chloroform, U.S.P., is washed with a small volume of dilute sodium bisulfite, and after redistilling twice on a water bath at 80° C., is stored in dark amber bottles.

Amyl alcohol is prepared by washing fusel oil or amyl alcohol, Tech., four times with equal volumes of water, finally discarding the wash water. The remaining solution is distilled on a hot plate, collecting the fraction boiling between 125° and 133° C. After redistilling a second time to increase its purity, it is stored in dark amber bottles.

Hydrochloric acid (0.5 per cent, 50 ml. of concentrated acid diluted with water to 2,000 ml.).

Permutit, according to Professor Otto Folin.

Phenol reagent, Folin and Denis.[14] This reagent is prepared as follows: 100 gm. sodium tungstate dissolved in 750 ml. water, 20 gm. phosphomolybdic acid, and 50 ml. 85 per cent phosphoric acid. The mixture is boiled for two hours, and after cooling is diluted to one liter.

Sodium Carbonate, C.P. (saturated solution).

The diazo mixture is prepared by mixing equal volumes of aqueous solutions of sodium sulphanilate (4 per cent), of hydrochloric acid (6 ml. concentrated acid, specific gravity 1.18 in enough water to make 100 ml.), and of sodium nitrite (1.4 per cent). The reagent should be freshly mixed and allowed to stand for five minutes before using.

Sodium hydroxide (10 per cent).

Ammonium molybdate, C.P. (10 per cent).

Sulfuric acid (2.5 Normal).

Ammonium vanadate, C.P. (2 per cent and filter).

A morphine standard containing 1 mg. of base per ml. solution is prepared by dissolving 0.3325 gm. purified morphine sulfate $(C_{17}H_{19}O_3N)_2H_2SO_4 + 5H_2O$ in 250 ml. water.

Extraction Procedure.—The quantitative removal of morphine from urine was carried out by the Pierce and Plant[1] procedure for dog urine. The procedure reported by them is as follows:

"Sixty cubic centimeters of fresh dog's urine are saturated with powdered sodium bicarbonate, giving a distinctly alkaline reaction to litmus. The alkaline urine is transferred to a liquid-liquid extractor in which 110 to 125 c.c. of a mixture of one part ethyl alcohol (95 per cent) and three parts chloroform has been placed, one-fourth the amount being in the extraction chamber, the rest in the extractor flask along with 0.5 c.c. of concentrated hydrochloric acid. Enough heat is applied to keep the solvent in active ebullition, and extraction continued for ninety to one hundred and twenty minutes. This is equivalent to treating the alkaline urine with 10 to 20 volumes of the solvent, and removes all of the morphine along with the small amounts of other substances. The alcohol-chloroform extract is transferred to an evaporating dish, together with several small portions of 0.5 per cent aqueous hydrochloric acid used to rinse the flask, and evaporated to dryness on a water bath.

The residue is then treated, first with 10 c.c., then twice with 5 c.c. of a 0.5 per cent hydrochloric acid, and the dish rinsed with two or three small portions of distilled water; the combined extracts and washings, usually about 35 c.c., are transferred without filtration to a 125 c.c. cylindrical separatory funnel and extracted three times with an equal volume of a mixture of one part amyl alcohol and three parts chloroform. During each of the extractions the funnel is shaken for five minutes in a mechanical shaker. The organic solvent is discarded and the aqueous solution saturated, in the funnel, with powdered sodium bicarbonate. The aqueous alkaline solution is then extracted three or four times with one and a half to two volumes of the amyl alcohol-chloroform mixture, shaking for five to ten minutes in each extraction. The combined amyl alcohol-chloroform extracts are then acidulated with dilute hydrochloric acid and evaporated to dryness on a water bath. Prolonged heating

of the dry residue from this or from the ethyl alcohol-chloroform extract should be avoided. The residue is then dissolved in a suitable amount of 0.5 per cent hydrochloric acid and the amount of morphine in it determined."

Fig. 1 is a photograph of the continuous liquid-liquid extractor having a maximum capacity of 70 ml. of urine of the form used by Balls and Wolff and modified by Pierce and Plant. For certain experimental studies we have used a larger liquid-liquid extractor, the extraction column of which was filled with glass beads to increase the surface area exposed to the extracting liquid. This

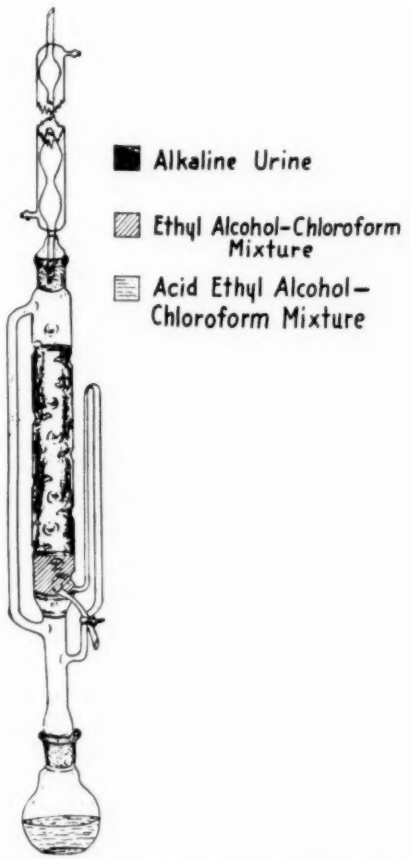

Fig. 1.—Continuous liquid-liquid extractor.

extractor so used has a maximum capacity of 150 ml. and separates satisfactorily very small amounts of morphine to be determined by the improved Deckert method. We have fitted these extractors with the interchangeable ground glass connections, made by Ace Glass Inc., which are especially desirable when dealing with small amounts of morphine in urine. Unless special high grade corks are available, considerable amounts of impurities are extracted from them. They also must be changed frequently, for the extraction reagents readily decompose them. Rubber stoppers are not very satisfactory, for they swell considerably after several extractions.

The Separation of Morphine From a Urinary Extract by Means of Permutit.—Four grams of permutit are placed in a 50 ml. volumetric flask and washed twice with distilled water, the supernatant fluid being discarded. The urinary residue obtained after the evaporation of the organic solvent is dissolved in 2 or 3 ml. of water and is transferred quantitatively by several washings to this flask. If insoluble matter is present after dissolving the residue with water, it must first be removed by filtration, the small filter paper being thoroughly washed four or five times and all wash water added to the permutit. This solution is frequently shaken several minutes with the permutit for about half an hour to allow the morphine to react with the permutit. After the permutit has settled to the bottom of the flask, the supernatant fluid is withdrawn by suction through a drawn tube or by decantation. The permutit is washed three times with 10-15 ml. portions of distilled water to remove all soluble impurities. The morphine concentration in the permutit is determined by any suitable colorimetric procedure.

Great care must be taken not to wash out any permutit from the flask. Flat bottom flasks, such as volumetric flasks, are much more suitable than graduated test tubes for the extraction of morphine by permutit, since they allow a larger surface area of permutit to be exposed to the liquid phase.

The dissolved urinary residue must be neutral when it is shaken with permutit. If the solution should be slightly too alkaline or too acidic, morphine will not be removed quantitatively by means of the permutit. Those bases whose "strength" is represented by a dissociation constant of 5×10^{-9}, or greater, are removed by permutit.[15] Ammonia reacts with permutit but does not give a color with any of the morphine color reagents, consequently enough permutit must be present to combine with both. Ordinarily 4 gm. of permutit are sufficient to combine with morphine in a residue up to a concentration of about 1.5 mg. per 65 ml. of urine. One gram of permutit will completely remove 1.0 mg. of morphine from a water solution.

The complete removal of morphine with permutit from a solution of urinary residue extracted by the regular procedure was tested by transferring the solution, after shaking with permutit, to a second flask containing permutit. The solution was again shaken with permutit, after which it was discarded. The permutit in each flask was washed three times with water and finally treated by the reagents used in the new colorimetric method. Color developed in the first flask as usual, but was absent in the second. A number of such tests indicated the complete removal of morphine in the residue by permutit in amounts up to about 1.5 mg.

A Colorimetric Method for the Determination of Morphine Using a Phenol Reagent.—The removal of morphine from fresh urine is carried out by the Pierce and Plant procedure, except that the acidulated residue from the liquid-liquid extractor is treated only once with the amyl alcohol-chloroform mixture. After treating the solution of urinary extract with permutit in the 50 ml. volumetric flask, the morphine concentration in the permutit is determined colorimetrically by the addition of 6 ml. saturated sodium carbonate and 2 ml. of Folin-Denis phenol reagent.[14] A blue color develops immediately which is quite stable for a day. The standards are prepared by the addition of known

amounts of morphine to 4 gm. of washed permutit in similar flasks, followed by treatment with sodium carbonate and phenol reagent. The flasks are filled to volume with distilled water, the contents thoroughly mixed and, after two or three hours, the unknowns are compared with the standard in a colorimeter. The use of a No. RG1 filter (red), made by Jena Glass Works, placed in the eyepiece of the colorimeter greatly facilitates the ease in matching the colors, especially if the blue color is very weak.

This color test is very sensitive for small amounts of morphine. Amounts as low as 0.05 mg., diluted to 50 ml., can be read in a colorimeter fitted with the No. RG1 filter. Smaller amounts of morphine in a correspondingly reduced volume can be determined, provided it is certain that the solution is practically free of other reducing substances.

Uric acid and phenols must be completely removed from a urinary residue, for they give a blue color with the phenol reagents. Since these substances do not react with permutit, they can be removed by washing.

TABLE I

RECOVERY OF MORPHINE ADDED TO URINE

The recovery of morphine added to urine using the liquid-liquid extraction process followed by further purification with acid and alkaline extractions. The color was developed by the addition of sodium carbonate and phenol reagent to the permutit.

URINE VOLUME	MORPHINE ADDED TO URINE	MORPHINE RECOVERED	EXPERIMENTAL ERROR
ml.	*mg.*	*mg.*	*mg.*
50	0.00	0.024‡	0.024
70	0.05	0.077	0.025
50	0.05	0.045	-.005
50	0.10	0.108	0.008
50	0.10	0.103	0.003
75*	0.00	0.240	—
75*	0.10	0.343	0.003
50	0.20	0.200	0.000
50	0.20	0.200	0.000
50	0.20	0.180	-.020
50	0.20	0.174	-.026
50	0.30	0.300	0.000
50	0.40	0.351	-.049
50	0.40	0.364	-.036
70	0.50	0.488	-.012
70†	0.50	0.450	-.050
70	0.70	0.640	-.054
65	0.80	0.079	-.001
70	1.00	0.990	-.010
70	1.50	1.530	0.030
50	2.00	1.980	-.020

*Urine from a morphine addict.
†Water solution extracted in liquid-liquid extractor.
‡Trace of color due to extracted impurities equivalent to 0.024 mg. morphine.

Morphine-free urinary extracts obtained from 65 ml. urine treated with permutit, followed by the addition of sodium carbonate and phenol reagent, gave a color in the final solution which, when compared with standards, was equivalent to 0.02-0.05 mg. morphine. Small quantities of morphine are unavoidably lost in the extraction process. This loss is nearly compensated by the small amount of color obtained from impurities carried over from the urine. Table I shows that varying amounts of morphine added to urine have been satis-

factorily recovered by this method. Table II is presented to show the concentration of morphine in the urine of two uncontrolled addicts. It is not intended that any conclusions concerning morphine excretion be drawn from this table. A detailed study on morphine excretion is in progress.

TABLE II

MORPHINE CONTENT OF URINE FROM MORPHINE ADDICTS

DATE	URINE VOLUME	MORPHINE BASE IN 70 ML. URINE	MORPHINE BASE PRESENT IN 24-HOUR-URINE SPECIMEN	PER CENT MORPHINE BASE EXCRETED IN URINE
	ml.	*mg.*	*mg.*	*per cent*
1/ 7/38	1425	0.320	6.25	5.19
1/ 8/38	1350	0.450	8.69	7.21
1/ 9/38	1375	0.279	5.48	4.55
1/10/38	825	0.685	8.07	6.70
1/11/38	800	0.953	10.90	9.06
1/12/38	950	0.408	5.54	4.60
1/13/38	1450	0.377	7.80	6.48
Addict 2. Intake 200 mg. morphine sulfate (150.4 mg. base) per day				
1/ 6/38	1600	0.375	8.58	5.70
1/ 7/38	1950	0.395	11.00	7.31
1/ 8/38	1350	0.500	9.65	6.42
1/ 9/38	1700	0.368	8.92	5.93
1/10/38	2050	0.321	9.40	6.25

Diazotization Method for the Determination of Morphine.—The diazo reaction used in the determination of 1-10 mg. morphine in a urinary residue prepared by the Pierce and Plant procedure is carried out by dissolving the residue in 2 ml. of water followed by 1 ml. of diazo mixture and 2 ml. 10 per cent sodium hydroxide. After standing one and one-half hours, the solution is diluted to 10 ml. and the color compared in a colorimeter with a standard morphine solution treated in a similar manner. One milligram of morphine is about the minimum quantity which gives a satisfactory color by the diazo reaction when diluted to a final volume of 10 ml.

A residue obtained from 50 ml. morphine-free urine treated by the above diazo reaction gave a similar red color which was too dark for a morphine-free urine. The color intensity was not constant for different morphine-free urines. The recovery of 1.0 mg. morphine added to 50 ml. of urine and extracted by the usual manner gave in one instance a value of 1.5 mg. when compared with a 1.0 mg. standard. Apparently there are present substances other than morphine in the urinary residue which give a color reaction with the diazo reagents.

An experiment was made on a solution of urinary extract in which a large portion of the interfering substances giving the diazo color reaction was removed from the morphine by means of permutit. After washing the permutit three times with water, the diazo color was developed by the addition of the diazo mixture and the 10 per cent sodium hydroxide. The entire contents were heated for fifteen minutes on a boiling water bath. The color obtained was much less than that from a similar residue not treated with permutit.

The presence of gas bubbles suspended in solution which collected on the walls of the colorimeter cups and prisms was frequently observed while making readings for morphine in urine. To determine whether or not the presence of these bubbles would cause significant variations, an experiment was carried out using a set of three 1.0 mg. morphine standards. After the addition of the diazo mixture and standing one and one-half hours, the colors did not match satisfactorily with each other. Another set of three standards prepared similarly, but without the preliminary one and one-half hours' standing, was heated fifteen minutes in boiling water. After cooling and adjusting the volume to 10 ml., the colors were compared with each other. They matched exactly with each other, but were nearly twice as strong as the unheated ones. No gas bubbles suspended in the solution were present. After the two sets of standards were allowed to stand for twenty-four hours at room temperature, they were again compared with each other. The color of the unheated standards compared exactly with each other and had become darker, nearly approaching those of the heated ones. The size and concentration of these bubbles appeared to vary considerably with time and temperature, thus causing variable readings on the same sample. In the absence of these bubbles in both the standard and unknown, reproducible colorimetric readings were obtained. The heating process nearly doubled the sensitivity of the method and cleared the solution of suspended gas bubbles which interfered with the colorimetric readings.

The Improved Deckert Method.—A. Qualitative Procedure. The presence of morphine in the urine of morphine addicts may rapidly be determined by making two extractions with ethyl acetate, followed by the formation of a morphine-molybdate-vanadate complex. Approximately 35 ml. of fresh urine is made alkaline by saturating it with U.S.P. sodium bicarbonate. After filtering, 25 ml. of the filtrate is measured into a 150 ml. separatory funnel. To this is added an equal volume of ethyl acetate. The two solutions are vigorously shaken for two or three minutes and then allowed to separate. The lower layer, which is urine, is drawn off into a beaker for a repeated extraction. Rotating the separatory funnel back and forth a few times causes small amounts of urine adhered to the walls of the funnel to settle to the bottom for removal from the ethyl acetate. Since it is not possible to remove all traces of urine from the separatory funnel by draining, it is best to remove the ethyl acetate solution by pouring it carefully from the top of the separatory funnel into a dry filter paper and collect the filtered ethyl acetate in an evaporating dish. Traces of urine carried over with the ethyl acetate solution are very objectionable and must be kept to a minimum. The separated urine is treated once again with ethyl acetate, which, when separated from the urine, is added to the first portion with about 0.2 ml. of 0.5 per cent hydrochloric acid and the entire volume evaporated to dryness.

The residue is dissolved in 1.5 ml. of distilled water and 0.2 ml. of 2.5 N sulfuric acid. To this is added 0.2 ml. of 10 per cent ammonium molybdate, which on standing about ten to twenty minutes, precipitates most of the impurities from the solution, but does not remove an appreciable amount of morphine. The precipitate is removed by filtering the fluid through wet Whatman No. 42 filter paper, about 30 mm. in diameter, into a graduated cylinder.

The evaporating dish and filter paper are washed with three 0.5 ml. portions of distilled water, the washings being added to the first filtrate. The total volume should not exceed 3.0 or 3.2 ml. Two drops (0.1 ml.) of 2 per cent filtered ammonium vanadate are thoroughly mixed with this clear filtrate. After standing for a short period of time, a turbidity sets which appears as a white, finely divided, granular substance, remaining in suspension for a long time. The degree of this turbidity is roughly proportional to the amount of morphine present. In the absence of morphine there may be a slight precipitate in suspension, which is clear and translucent, but when examined by direct light at an oblique angle does not appear white and finely divided. Amounts of morphine as low as 0.03 mg. in 25 ml. of urine can readily be detected.

Occasionally a urine will form an emulsion with ethyl acetate which does not separate readily even after long standing. The emulsion can best be broken up by filtering it through a dry filter paper. If not all of it passes through the filter, the remaining portion is transferred to another dry filter paper. Ethyl acetate will not pass readily through a wet filter paper.

The original Deckert method was modified, after a number of attempts to detect the presence of small amounts of morphine had failed. When larger amounts of the drug were present, a positive result was obtained. Studies were then carried out to determine the factors responsible for changes in the sensitivity of the method. In addition to morphine large quantities of impurities are extracted by the ethyl acetate which are precipitated by ammonium molybdate and ammonium vanadate. These impurities must be separated from morphine as well as possible to prevent them from masking the morphine reaction. This separation was accomplished by precipitation of the impurities with ammonium molybdate at the isoelectric point followed by filtration. Morphine molybdate is not precipitated in concentrations ordinarily found in these experiments nor is an appreciable amount lost with the precipitate by absorption. Nitric, hydrochloric, and sulfuric acids were tested in the separation of these impurities and for the formation of the morphine-molybdate-vanadate complex. The best results were obtained by dissolving the urinary residue in 1.5 ml. water, followed by the addition of 0.2 ml. 2.5 N sulfuric acid. After adding ammonium molybdate, filtering, and washing the filter paper, there must be a definite acid concentration in the total filtrate to obtain the maximum separation of the morphine-molybdate-vanadate complex by the addition of ammonium vanadate. Excess acid in the solution increases the solubility of the complex. It appeared that with sulfuric acid the morphine-molybdate-vanadate complex was more finely divided and remained longer in suspension. Varying the concentration of the ammonium molybdate had no apparent effect on the separation of impurities or on the formation of the morphine-molybdate-vanadate complex. It is necessary that the final volume does not exceed 3.0 to 3.2 ml., or otherwise less morphine-molybdate-vanadate complex will separate out. After the complex has separated, the solution may be diluted to a volume suitable for nephelometric measurements.

Tests were made to show the relatively high concentration of morphine-molybdate-vanadate complex which never separates out of solution. After allowing the turbid solution to set for several days, it was centrifuged, and the supernatant

fluid removed by decanting. Both the supernatant fluid and the precipitate were tested with sodium carbonate and phenol reagent. When the final volumes in both were made equal, the color in the supernatant fluid was darker than that from the precipitate. No color was obtained from the reagents in the absence of morphine. The evidence is clear that the amount of morphine precipitated as the morphine-molybdate-vanadate complex depends on a number of factors, such as the acid concentration, the volume in which it is precipitated, the amount of impurities not removed, and the temperature of the solution. These factors must be carefully controlled before the method can be used satisfactorily as a quantitative method.

No turbidity has been obtained in any sample of urine free of morphine or other commonly used drugs. The presence of quinine in urine gives a turbidity which separates out immediately as a yellow, flocculent precipitate. Pyramidon (4-dimethyl-amino-antipyrine) gives a turbidity similar to morphine. After standing for a number of hours, pyramidon produces a blue green to blue color, which is not obtained by morphine alone. If pyramidon is suspected, additional tests should be made for its identification. The above test for the detection of morphine in urine should always be confirmed by one or more additional tests. The Marquis or Fröhde reagents give characteristic color reactions for morphine when added to a residue obtained by extracting an alkaline urine with ethyl acetate or other organic solvents.

B. Quantitative Procedure. This principle may be used quite satisfactorily in determining semiquantitatively very small amounts of morphine. A series of test tube standards were prepared by adding varying amounts of

TABLE III

TURBIDITY OBTAINED FROM VERY SMALL AMOUNTS OF MORPHINE

AMOUNT OF MORPHINE ADDED TO 25 ML. URINE	APPEARANCE OF FINAL SOLUTION
mg.	
0.00	Clear
0.01	Clear
0.02	Very faint trace
0.03	Definitely positive
0.05	Strong
0.07	Stronger
0.09	Much stronger
0.12	Very strong

morphine to 25 ml. of morphine-free urine and then extracting each sample twice with ethyl acetate, followed by the usual preparation of the morphine-molybdate-vanadate complex. The amount of morphine present in the urine of most addicts was so high that 10 ml. urine was usually sufficient for a satisfactory comparison with a prepared standard. The unknowns were allowed to stand at least two hours before they were compared with a standard. When the turbidity was very high, it was either diluted sufficiently to match with an appropriate standard, or a stronger standard was prepared. If a turbidity is very high, it is not easy to determine small differences in morphine concentrations. Table III shows the relative turbidity obtained by using very small amounts of morphine.

Further attempts were made to make the turbidity method quantitative. The procedure for morphine extraction described by Pierce and Plant was used in the following experiments. The final residues were treated similarly to those obtained from the ethyl acetate extractions. The morphine-molybdate-vanadate complex separated out as fine, white particles which remained in suspension for a long period of time. The presence of excess amounts of impurities caused the morphine compound to coalesce and precipitate faster. Efforts were made to compare the turbidity with an aqueous morphine standard similarly prepared, using a Bausch and Lomb nephelometer and a "Cenco" telophotometer. One hour after the addition of ammonium vanadate the final volume was diluted to 10 ml. and compared with a standard. Satisfactory results were obtained for very small amounts of morphine (Table IV).

TABLE IV

RECOVERY OF MORPHINE ADDED TO URINE

URINE VOLUME	MORPHINE ADDED TO URINE	MORPHINE RECOVERED	EXPERIMENTAL ERROR
ml.	*mg.*	*mg.*	*mg.*
50	0.00	0.000	0.000
50	0.05	0.050	0.000
50	0.10	0.097	-0.003
50	0.10	0.098	-0.002
100	0.10	0.099	-0.001
150	0.10	0.091	0.000
50	0.10	0.098	-0.002
50	0.10	0.088	-0.012
25	0.10	0.095	-0.005
65	0.10	0.100	0.000

The recovery of morphine added to urine by means of the liquid-liquid extraction process followed by further purification with acid and alkaline extraction. The morphine-molybdate-vanadate complex was prepared as usual. After standing for forty-five minutes, the volume was adjusted to 10 ml., and a comparison made with a similarly treated aqueous morphine standard by means of a nephelometer.

When the amount of morphine exceeded 0.25 mg., unsatisfactory results were obtained. The morphine-molybdate-vanadate particles clumped together into large particles which precipitated rapidly. Under such conditions it was not possible to make satisfactory comparisons with a standard. The turbidity obtained from urine specimens always precipitated more rapidly than that from aqueous morphine solutions.

Attempts were made to stabilize the turbidity by the addition of ghatti gum or arabic gum. Both of these substances prevented the formation of any turbidity.

When ordinary stopcock grease was used as the lubricant on the extractor and separatory funnels, low recoveries were often obtained. When the lubricant was replaced by glycerol, less morphine was lost. Considerable grease was always extracted by the organic solvents and was carried over in the final residue, thus preventing the complete transfer of morphine from the evaporating dish. Small amounts of glycerol do not interfere with the formation of the turbidity.

SUMMARY

1. Morphine may be determined quantitatively in concentrations ranging from 0.08 to 3.0 mg. per 100 ml. urine by a new colorimetric method. The morphine is extracted from the urine by the Pierce and Plant procedure, using the continuous liquid-liquid extraction process followed by acid and alkaline extractions. The final residue dissolved in water is further purified by means of permutit. Morphine combines with permutit, while most other reducing substances are removed by washing. The morphine is finally determined colorimetrically after the addition of sodium carbonate and Folin-Denis phenol reagent to the permutit.

2. The determination of morphine in urine by the diazo reaction has been modified and its limitations discussed.

3. The presence of morphine in the urine of morphine addicts may rapidly be determined by making two extractions with ethyl acetate and the subsequent production of a morphine-molybdate-vanadate complex. A turbidity appears which is roughly proportional to the morphine concentration and may be compared with a prepared standard. Morphine concentrations as low as 0.03 mg. in 25 ml. of urine can readily be detected.

4. The turbidity produced by the morphine-molybdate-vanadate complex has been developed into a quantitative method for small amounts of morphine extracted from urine by the Pierce and Plant procedure. A nephelometer is used for comparing the unknown turbidity with a standard.

REFERENCES

1. Pierce, I. H., and Plant, O. H.: Studies of Chronic Morphine Poisoning in Dogs. IV. Excretion of Morphine in Tolerant and Non-tolerant Animals, J. Pharmacol. & Exper. Therap. **33**: 201, 1932.
2. Lautenschläger, L.: Die Diazoreaktion des Morphiums, Arch. d. Pharmazie **257**: 13, 1919.
3. Balls, A. K., and Wolff, W. A.: The Determination of Morphine, J. Biol. Chem. **80**: 379, 1928.
4. Deckert, W.: Ein neues, leicht ausführbares Schnellverfahren zur quantitativen Bestimmung kleinster Mengen von Morphin in Harn, Blut und anderem biologischen Material, Arch. f. exper. Path. u. Pharmakol. **180**: 656, 1936.
5. Fry, E. G., Light, A. B., Torrance, E. G., and Wolff, W. A.: Opium Addiction, The Excretion of Morphine by Human Addicts, Arch. Int. Med. **44**: 862, 1929.
6. Wolff, W. A., Riegel, C., and Fry, E. G.: The Excretion of Morphine by Normal and Tolerant Dogs, J. Pharmacol. & Exper. Therap. **47**: 391, 1933.
7. Loofs, F. A.: Ueber die Möglichkeit einer Klinisch-Chemischen Kontrolle des Morphinismus. Bemerkungen zur Theorie der Morphin-Gewohnung, Ztschr. f. d. ges. Neurol. u. Psychiat. **79**: 433, 1922.
8. Panse, F.: A Simple, Reliable Method for the Detection of Morphine in the Urine of Addicts, Fortschr. d. Therap. **8**: 629, 1932.
9. Teruuchi, Y., and Kai, S.: On the Fate of Morphine Which Has Been Injected Into the Animal Body, J. Pharmacol. & Exper. Therap. **31**: 177, 1927.
10. Ellinger, P., and Seeger, H.: The Determination of Morphine in Excretions, Secretions, and Tissues, Arch. f. exper. Path. u. Pharmakol. **174**: 160, 1933.
11. To, S., and Rin, A.: The Determination of Morphine in the Urine of Opium and of Morphine Addicts and the Practical Application of the Reaction, Jap. J. M. Sc., IV, Pharmacol. **7**: 11, 1935.
12. Trehden, O., and Huang, C. H.: Microdetection of Morphine and Heroine in Urine and Milk by Spot Method, Pharm. Monatsh. **18**: 73, 1937.
13. Giang, C. K.: A New Method for the Detection of Morphine in the Urine of Opium Addicts, Chinese M. J. **51**: 211, 1937.
14. Folin, O., and Denis, W.: On Phosphotungstic-Phosphomolybdic Compounds as Color Reagents, J. Biol. Chem. **12**: 239, 1912.
15. Whitehorn, J. C.: "Permutit" as a Reagent for Amines, J. Biol. Chem. **56**: 751, 1923.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

TUBERCULIN TEST, Intracutaneous, Paretsky, M. Am. Rev. Tuberc. 38: 81, 1938.

A series of 400 "double" tests has demonstrated that the regional variation of specific skin hypersensitiveness to tuberculin is a factor of a practical significance.

A series of 500 "double" tests has yielded results seemingly indicating that the deterioration of tuberculin does not affect its potency to a degree definitely distorting the outcome of the intracutaneous reaction.

A series of 400 "double" tests has revealed that the "tenacity" of tuberculin undoubtedly exerts a distorting effect on the result of the intracutaneous reaction, depicted in our series by a curve which reached its peak when the 1:100,000 solution of old tuberculin was used, and which came down to its lower levels with the increase of the concentration of the solutions of old tuberculin.

A series of 100 "double" tests has apparently failed to demonstrate a noticeable effect of the size of the wheal produced by the injected tuberculin on the size of the reacting skin area.

A series of 100 "double" tests has given results suggesting a probable somewhat retarding effect of alcohol on the intracutaneous tuberculin reaction.

PNEUMOCOCCUS TYPING, Concentration Method Applicable to the Neufeld Quellung Reaction in Sputums, Taplin, G. V., Meneely, G. R., and Hettig, R. A. J. A. M. A. 111: 410, 1938.

1. The entire specimen of sputum is emulsified by drawing it up in a small (2 c.c.) syringe and mixing it thoroughly in a sterile Petri dish until all large particles are broken up.

2. Into a sterile centrifuge tube is placed 0.5 c.c. of the emulsion, and from 6 to 10 c.c. of physiologic solution of sodium chloride is added, depending on the viscosity of the sputum. This mixture is emulsified by drawing the diluted specimen back and forth in a sterile 10 c.c. pipette.

3. The emulsified specimen is put in the centrifuge at 1,000 r.p.m. for two or three minutes to remove cells and detritus, the bacteria remaining in the supernatant fluid.

4. The supernatant fluid is transferred to another tube, which is put in the centrifuge at 3,000 r.p.m. for ten minutes, after which the clear fluid is poured off, leaving about 0.25 c.c. above the sediment.

5. The bacterial sediment is resuspended in the small remnant of supernatant fluid by shaking, and the resulting suspension is used for the typing tests. Before the suspension is mixed with specific typing sera, two drops of methylene blue are added and mixed with this suspension, the organisms being allowed to stain before the Quellung reaction takes place. This has been used because in many cases when the sera and stains are added simultaneously, the organisms swell before they stain and may be difficult to recognize.

As a result of this procedure, the Quellung reaction takes place quickly, in some cases, immediately. Detection of capsular swelling is rendered much easier by the lack of cells and detritus and by the larger number of organisms in the field (from 10 to 300 per oil immersion field). No injury to the capsule has been observed as a result of the dilution with physiologic solution of sodium chloride and centrifugation. No false positive reactions have been observed. The suspension and washing of sputum in saline solution probably removes the soluble substance that causes agglutination, and failure of the Quellung reaction in other highly concentrated specimens. With this technique highly concentrated suspensions may agglutinate but they never fail to swell.

GONOCOCCI, A New Contrast Stain for Meningococci and, Sandiford, B. R. Brit. M. J. May 28, p. 1155, 1938.

Any Gram technique to which the worker is partial may be followed up to the end point of decolorization, but the following is strongly recommended, especially the use of acetone, which is essential in order to obtain complete decolorization of the cell nuclei before counterstaining.

Initial Stain

Crystal violet	1 gm.
Alcohol (98 per cent)	20 c.c.
Ammonium oxalate (1 per cent aqueous)	30 c.c.

1. Leave this mixture on the heat-fixed film for thirty seconds.
2. Flood off with triple strength Lugol's iodine (as used in Jensen's modified Gram stain) and leave on for thirty seconds.
3. Pour off the excess iodine and blot once.
4. Decolorize with acetone for three or four seconds.
5. Wash.
6. Put on the counterstain for two minutes.
7. Flood off with water—do not wash—and blot.

Contrast Counterstain

Malachite green	0.05 gm.
Pyronine	0.15 gm.
Distilled water	100.0 c.c.

Note: There are two varieties of pyronine "B" and "G," the former being almost insoluble in water. Pyronine (Grubler) is apparently pyronine "G," as it is freely soluble in water.

Keeping Qualities: The following statements are based on haphazard observations only, during the use of solutions kept in ordinary bottles at room temperatures under 20° C.: Crystal violet stain keeps for about a month and deteriorates after six weeks. Counterstain keeps for at least three weeks, possibly longer; 100 c.c. in a staining pot may be used for at least 150 small smears.

TUMORS, Multiple Primary Malignant, Eisenstaedt, J. S. J. A. M. A. **110**: 2056, 1938.

A man who died in 1936 had been operated upon twenty-one years before for adenocarcinoma of the kidney. He later was affected with two other primary cancers in distinct and separate organs, one of which, that of the prostate, produced metastases to the lungs and lymph nodes. All three cancers were diagnosed clinically.

It is the author's belief, after a rather complete survey of the literature, that:

1. Multiple primary cancers are more frequent than are reported.
2. They occur more frequently than chance alone would explain.
3. The percentage of multiple primary malignant tumors to be reported in the future will be higher than that recorded in the past, because:

(a) More people are reaching advanced years, a period in which the incidence of cancer is generally high. (b) Since the inauguration of cancer clinics and commissions throughout the world, cancer patients have been more concentrated and more thoroughly studied. (c) Better results are being obtained in the treatment of cancer than heretofore. (d) As a result of longer survival after treatment for a single primary tumor, time is afforded for the development of subsequent primary cancers. (e) Autopsies are more widely done and with greater thoroughness than in the past.

4. Some factor, as yet unknown, possibly hereditary or hormonal in nature, plays an important part in susceptibility to malignant disease, and the varied responses to environment in different individuals depend on this unknown element.

TISSUE: Staining of Acid-Fast Bacilli in Paraffin Sections, Fite, G. L. Am. J. Path. **14:** 385, 1938.

The optimal staining of acid-fast bacilli in tissues involves fixation in an alcoholic medium, removal of mercuric deposits with iodine followed by alcohol and sodium thiosulfate, and staining in a 1 per cent solution of new fuchsin in 5 per cent phenol and 10 per cent methyl alcohol.

If a potassium dichromate fixative is used, the period of fixation must be brief and the tissue thoroughly washed, and the section must be treated with potassium permanganate and oxalic acid.

Time and materials are wasted in trying to stain acid-fast bacilli in tissues subjected to prolonged passage in formaldehyde or decalcifying fluids.

To ensure the maximal staining possible under the conditions of fixation, the sections should be stained:

After alcohol fixation, at 20° C.—two to eight hours,
at 37° C.—one to four hours,
at 60° C.—thirty minutes to two hours, or
at 90° C. (steaming)—five minutes;

After all other fixation, at 20° C.—sixteen to twenty-four hours,
at 37° C.—twelve to sixteen hours,
at 60° C.—eight to twelve hours, or
at 90° C.—five minutes.

Under good conditions bacilli may be adequately stained in much shorter periods of time than these. Bacilli not stained by fuchsin in the maximum times given will not be stained by further treatment. This is the point to which the duration of staining is best carried. Prolonged staining at room temperature is preferred.

New fuchsin as a dye for acid-fast bacilli is much superior to basic fuchsin.

When, in Zenker fixed tissues, fuchsin fails to stain bacilli resistantly, they may sometimes still be stained by Gram's stain, by the acid-fast method using crystal violet, or by the fuchsin-formaldehyde method here recorded.

The following stain is recommended:

New fuchsin	1 gm.
Phenol crystals	5 gm.
Methyl alcohol	10 c.c.
Distilled water to make	100 c.c.

The dye is wholly dissolved in the mixture of alcohol and phenol, and the distilled water is added, not too rapidly. The solution should be clear and not require filtration. Fuchsin is so readily precipitated by a wide variety of substances that to maintain the full strength distilled water of high purity must be used. The solution preserves indefinitely, but it should be remembered that evaporation of 5 per cent by volume, which will largely be alcohol, will precipitate dye to a much greater proportion. Solutions left in Coplin jars lose strength rapidly.

TUBERCULOSIS, Bromsulphalein Test and Blood Cholesterol in Pulmonary Tuberculosis, Levinson, S. A., and Siegal, H. A. Am. Rev. Tubere. **38:** 229, 1938.

The bromsulphalein liver function test and blood cholesterol were studied in 28 cases of far-advanced pulmonary tuberculosis. These tests can be used as an index in the prognosis of the disease condition.

The combination of hypocholesterolemia with bromsulphalein retention indicates extreme gravity.

The hypocholesterolemia and bromsulphalein retention may be related to the functional activity of the reticulo-endothelial system, as evidenced by the greater demand for cholesterol by the organ. The degree of stimulation of the reticulo-endothelial system can be measured by the amount of bromsulphalein dye retention. The greater the stimulation the greater the retention of the dye.

TUBERCULIN: Mantoux Tests With the Gottschall-Bunney Diluent for, Clark, E. Am. Rev. Tubere. **38**: 270, 1938.

A total of 1,648 Mantoux tests were performed from December 15, 1936, to March 19, 1937, comparing tuberculin freshly diluted in saline with that ready diluted in the diluent of Gottschall and Bunney.

The results of these tests seem to indicate that the ready diluted tuberculin is as efficient as the freshly diluted tuberculin and retains its potency when kept at room temperature for a period of at least one month.

Tuberculin ready diluted 1:10,000 and incubated thirty days at 37° C. showed some deterioration evidenced by a difference in induration of the Mantoux reaction. Although the sizes of the reactions were comparable, the induration was not quite as hard as that caused by freshly diluted material.

RETICULOCYTES, Demonstration of, With Wright's Stain, Kitchen, S. F. Stain Technol. **13**: 107, 1938.

A 0.3 per cent solution of the stain in absolute, acetone-free methyl alcohol was used, and in making moist preparations a thin, even film of the dye was spread on a glass slide. A small drop of blood was placed on a cover slip which was then inverted on the slide and the preparation sealed with vaseline.

The reticulocytes showed up quite as numerously as they did when brilliant cresyl blue was similarly applied, although a little more slowly. In such preparations the reticulum appeared not infrequently in the form of filaments which usually had a tendency to interlace, sometimes quite densely, so that there was a compact mass in the center of the red cell, or perhaps eccentrically placed. At other times the interlacing network was more loosely formed and traversed the cell like a band. Most frequently the reticulum was observed to assume a different morphologic character. This was a branching form which gave it an appearance not unlike moss. In such instances it was fuzzy and much less well defined. A reticulocyte might exhibit as little as a single small Y-shaped branch, or the branching forms might be scattered quite profusely throughout the cell. Regardless of what form the filamentous reticulum assumed, one or more metachromatic globules, varying considerably in size and exhibiting Brownian movement, were occasionally observed. In some instances these globules were isolated and at other times in intimate relationship with the filamentous material.

Dry slide preparations were made by spreading a moderately heavy film of the 0.3 per cent dye solution on a glass slide, and when this was dry, superimposing a blood smear thin enough so that the individual cells were separated. Wright's stain was then used as a counterstain in the usual way. In these preparations the reticulum usually appeared as granules of varying size and number. The intensity of the staining reaction varied greatly, as well as the amount of reticulum so precipitated. In some cells there was observed a light sprinkling of very fine granules, while others were packed with heavily-stained, short, thick rods. Still other reticulocytes might contain a relatively small number of larger, coarse granules which were frequently irregular in outline.

HEPATOSPLENOGRAPHY: Histopathologic Study of Tissues of 65 Patients Injected With Thorium Dioxide Sol for, Yater, W. M., and Whitmore, E. R. Am. J. M. Sc. **195**: 198, 1938.

Necropsies have been performed on 64 patients at intervals of a few days to three years after the injection of an average dosage of 75 c.c. of thorotrast for the purpose of making hepatosphenograms. In no case was there any evidence of injury to the tissues nor cellular reaction that could be ascribed to the presence of the thorium dioxide.

A subcutaneous nodule was excised from the arm four years and five months after the injection of thorotrast accidentally into the subcutaneous tissues. Thorium dioxide was walled off by dense hyaline connective tissue, the nodule resembling those found in the lungs in nodular silicosis. Nowhere was there any evidence of injury to the tissues nor cellular reaction other than the primary reaction resulting in the walling off of the thorium dioxide.

REVIEWS

Books and Monographs for Review should be sent direct to the Editor, Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

Digrafía*

THIS monograph describes a new method of roentgenography for the study of moving organs. The principle involved is briefly a series of narrow parallel slats which completely cover a film, and which are hinged at the ends in such a way that every other slat may be opened at a given time for one exposure, and at any desired interval thereafter the other half of the slats are opened to make the second exposure. One half may, therefore, be open during systole of the heart and the other half during diastole—both of these being, therefore, recorded on the same film, permitting accurate comparison of area, range of motion, etc. The principle can be extended in such a way as to include even more exposures on the same film. The method would appear to have distinct possibilities and certain definite advantages over roentgen-kymographs.

Sulfanilamide Therapy of Bacterial Infections†

PERHAPS, not since the discovery of salvarsan has the introduction of a new chemotherapeutic agent aroused more interest than that following the introduction of sulfanilamide.

Despite the fact that its mode of action and its limitations are still far from clearly understood, that this compound possesses remarkable potentialities is becoming more and more evident so that this volume, representing a careful survey by workers of experience, is one to be read with the greatest of interest and profit.

The book begins with a comprehensive survey of the literature dealing with the sulfonamide compounds, including a survey of their experimental and clinical use. This is followed by a section dealing with the authors' in vitro experimental studies of sulfonamide and their results in the treatment of streptococcal and pneumococcal infections in animals and their experience in the treatment of pneumonia and streptococcal meningitis in man.

Section III discusses the mechanism of the action of sulfonamide. Section IV discusses general therapeutic considerations and the criteria of therapeutic efficiency.

An addendum of 18 pages reviews studies which appeared after the manuscript went to press.

While presenting a comprehensive survey of the literature it must not be thought that this book is a mere compilation of abstracts. On the contrary, the authors on every page show evidence of ability to evaluate the studies of others as well as their own and of a clear concept of the complexities involved in the study and evaluation of chemotherapy.

This is not only a timely book, a thoughtful book and one presenting a well-balanced consideration of experimental and clinical evidence; it is a valuable contribution of marked importance.

This book should be read by all who are interested in the chemotherapeusis of disease.

---

*Digrafía. By Guido Pollitzer, M.D. 104 pages, 41 illustrations. Imprenta Amorrortu, Buenos Aires.

†Sulfanilamide Therapy of Bacterial Infections, With Special Reference to Diseases Caused by Hemolytic Streptococci, Pneumococci, Meningococci and Gonococci. By Ralph R. Mellon, M.D., Director, Institute of Pathology, Western Pennsylvania Hospital; Paul Gross, M.D., Pathologist to the Institute; and Frank B. Cooper, M.S., Research Chemist to the Institute. Cloth, 398 pages, $4.00. Charles C. Thomas, Springfield, Ill.

The Foot*

THAT this book should have reached a second edition is not surprising, for it presents in an eminently simple and practical manner a discussion of ailments, the importance of which has not always been emphasized to the practitioner.

The present edition is enriched by a considerable increase in the number of illustrations, and also by a revision and addition to the text.

As before, this book may be recommended as one of practical value to the practitioner.

Handbook of Practical Bacteriology†

THIS deservedly popular English text remains, as before, an eminently practical guide to bacteriologic laboratory work and as such can be well recommended.

It has been thoroughly revised to embody the changes and advances which have taken place in the four years since the last edition.

Clinical Chemistry and Practical Medicine‡

IT IS not surprising to find that this book has reached a second edition, for it is an excellent and practical exposition of particular value to the practicing physician.

Biochemistry has become an important and almost indispensable adjunct to clinical medicine. In this book the physician will find a clear-cut and comprehensive discussion of its practical application to the problems of everyday medicine.

Clinical Atlas of Blood Diseases§

THOUGH small in size, this volume contains a wealth of readily assimilable information and that it has reached a fourth edition is not surprising.

Five new plates have been added in the present edition, and the text has been revised in accordance with the advances in the field of hematology.

The physician, the pathologist, and the laboratory worker will find this book a valuable addition to the working reference library.

It can be recommended with enthusiasm.

The Troubled Mind¶

AS STATED in the preface, the purpose of this book is to present in simple terms a description of mental and nervous diseases, with particular emphasis on the psychopath as a disturber of the public peace.

---

*The Foot. By Norman C. Lake, M.D., M.S., D.Sc., F.R.C.S., Senior Surgeon and Lecturer on Surgery, Charing Cross Hospital, etc. Cloth, ed. 2, 366 pages, 113 illustrations, $4.50. William Wood & Co., Baltimore, Md.

†Handbook of Practical Bacteriology. By T. J. Mackie, M.D., D.P.H., Professor of Bacteriology, University of Edinburgh; and J. E. McCartney, M.D., D.Sc., Director of Research and Pathological Services, London County Council. Cloth, 586 pages, $4.00. William Wood & Co., Baltimore, Md.

‡Clinical Chemistry and Practical Medicine, By C. P. Stewart, Lecturer in Biochemistry, University of Edinburgh; and D. M. Dunlop, Christison Professor of Therapeutics and Clinical Medicine, University of Edinburgh. Cloth, 372 pages, 38 illustrations, $4.00. William Wood & Co., Baltimore, Md.

§Clinical Atlas of Blood Diseases. By A. Piney, M.D., M.R.C.P., Consulting Physician, International Clinic, Tunbridge Wells, etc.; and Stanley Wyard, M.D., M.R.C.P., Physician, the Royal Cancer Hospital, London, etc. Cloth, ed. 4, 127 pages, 42 illustrations, 38 in color, $4.50. P. Blakiston's Son & Co., Philadelphia, Pa.

¶The Troubled Mind. A Study of Nervous and Mental Diseases. By C. S. Bluemel, M.A., M.D., F.A.C.P., M.R.C.S. (Eng.). Cloth, 520 pages, $3.50. Williams & Wilkins Co., Baltimore, Md.

The book is divided into eight main sections: I. Fixed Ideas and Reactions; II. Psychoneurosis—Its Manifestations; III. Psychoneurosis—Its Nature and Causes; IV. Traumatic Hysteria; V. Clinical Types of Inhibition; VI. Sundry Disorders; VII. Mental Illness; and VIII. The Patient's Role in Therapy.

It is quite evident from the text that the author has had an extensive and comprehensive experience, and also that he has the ability to discuss a complex subject in a clear and understandable fashion.

A glossary is appended for the lay reader.

Those who read this book will undoubtedly have a better understanding of the mental mechanism and a clearer appreciation of the disturbances to which it is subject.

A Textbook of Bacteriology*

THIS second edition, written in considerably fewer pages than the average textbook on bacteriology, seeks to present only the fundamental and definitely accepted phases of the subject. The book is concise, practical, and sufficiently complete for the beginner. This is quite in keeping with the feeling of the author: that the beginning student or practitioner cannot assimilate the subject matter given in the more inclusive textbooks.

Pathological Technique†

EVERY laboratory must appreciate the appearance of Dr. Mallory's new presentation of pathologic techniques.

"I have undertaken the writing of this book as a result of many requests for a modern presentation of accepted pathological techniques," Dr. Mallory writes in beginning the preface to the book. "Technical methods are constantly changing in pathology as in other branches of science. The wisest plan in issuing a book like this seems to be to retain the best of the past and to add only the most promising methods of the present." The author has done just that, and in the same degree of excellence that characterized the old Mallory and Wright of more than a decade ago. The new publication retains only the accepted techniques of the past, and includes the more modern procedures which up until now have been accessible only in the foreign literature or individual publications.

For years the pathologic laboratory has felt the need for such a book as Dr. Mallory's, and a cordial welcome is assured.

General Bacteriology‡

A COMPREHENSIVE textbook of general bacteriology intended especially for the medical student. The fact that the book has reached a twelfth edition speaks for its usefulness. Dr. Burrow's revision considers the field of bacteriology from the broader point of view, especially clinically, leaving, as indicated in the preface, the more special laboratory procedures to manuals of bacteriology.

---

*A Textbook of Bacteriology. By Thurman B. Rice, A.M., M.D., Professor of Bacteriology and Public Health, Indiana University School of Medicine. Cloth, 554 pages, $5.00. W. B. Saunders Co., Philadelphia, Pa.

†Pathological Technique. By Frank Burr Mallory, A.M., M.D., S.D., Consulting Pathologist to the Boston City Hospital. Cloth, 397 pages, $4.50. W. B. Saunders Co., Philadelphia, Pa.

‡General Bacteriology. A Textbook. By Edwin O. Jordan, Ph.D., Formerly Professor of Bacteriology, University of Chicago. Revised by William Burrows, Ph.D., Assistant Professor of Bacteriology, University of Chicago. Cloth, ed. 12, 825 pages, $6.00. W. B. Saunders Co., Philadelphia, Pa.